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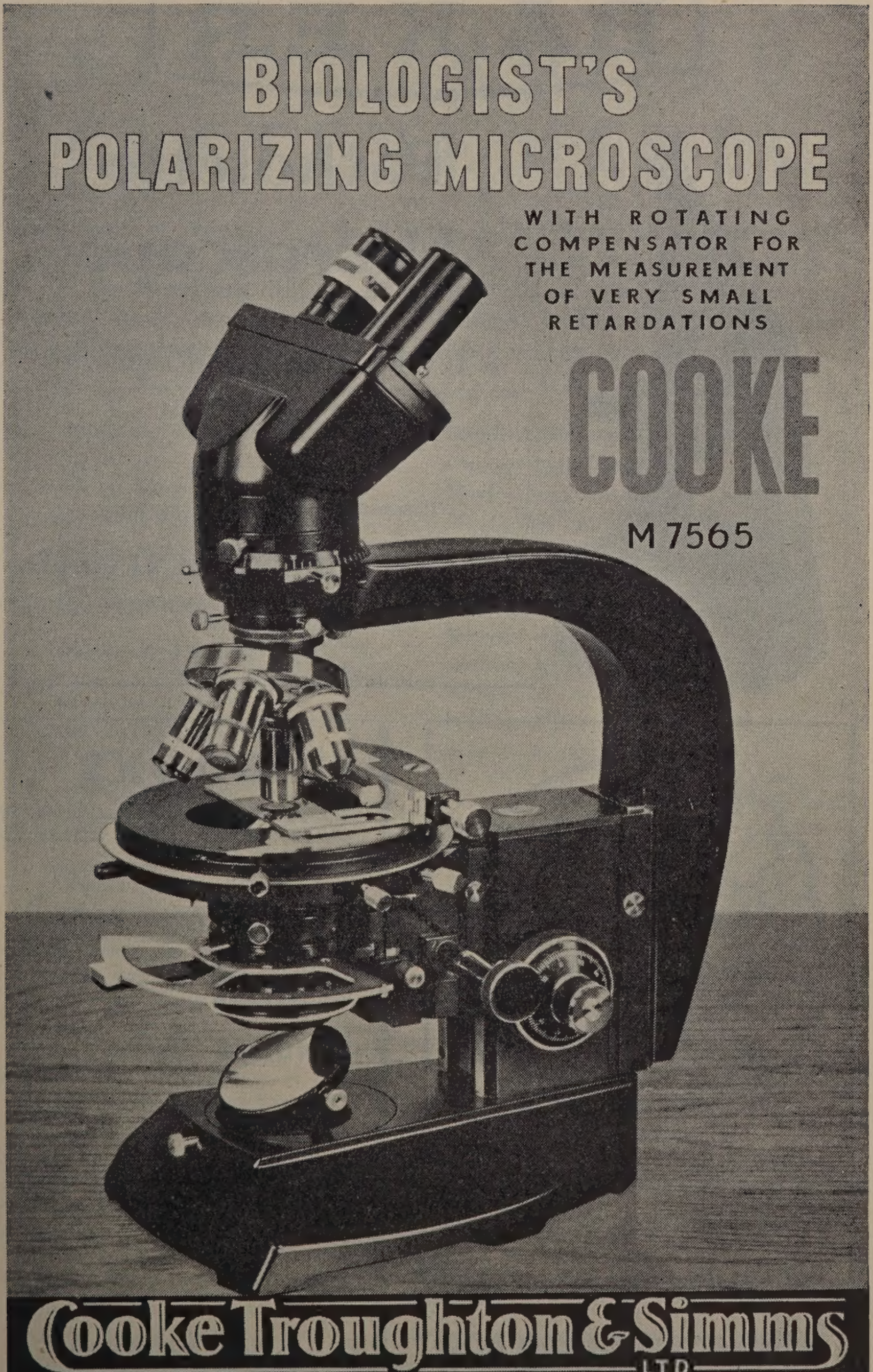

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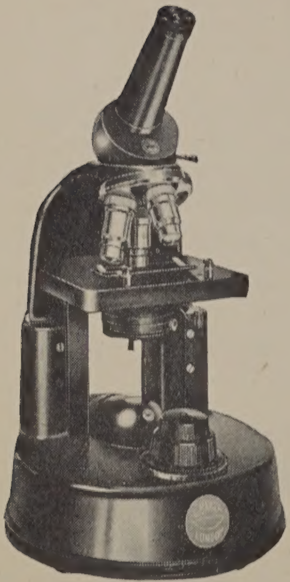

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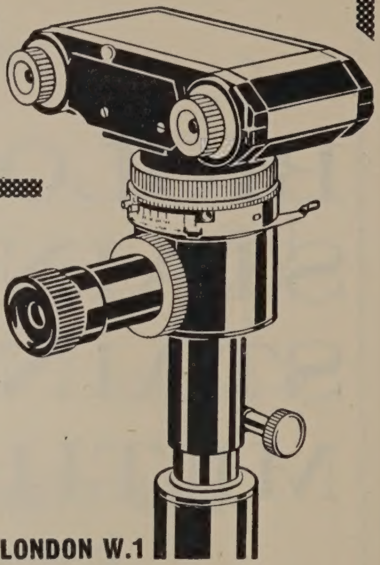

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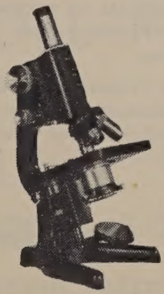

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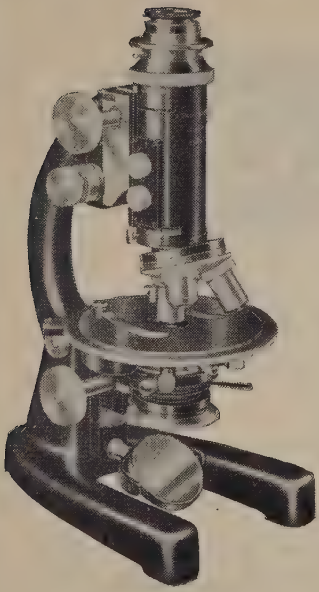

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The Structure and Mode of Functioning of the Reproductive Organs of *Tritonia hombergi* (Gastropoda Opisthobranchia)

By T. E. THOMPSON

(*From the Marine Biological Station, Port Erin, Isle of Man. Present address: Zoology Department, University College, Cardiff*)

With one plate (fig. 4)

SUMMARY

Routine observations on *Tritonia hombergi* Cuvier dredged from an area off the south coast of the Isle of Man indicate that the species has an annual life-cycle similar to that of many dorid nudibranchs.

The ovotestis communicates with the anterior genital mass through numbers of collecting tubules which unite to form a stout hermaphrodite duct. This duct functions as a vesicula seminalis in adult specimens; spermatozoa stored here are physiologically immature. The hermaphrodite duct bifurcates to form male and female ducts; the region of the bifurcation forms a valve ensuring that oocytes shall not enter the purely male regions of the genital system during oviposition. The male duct is the glandular and muscular vas deferens which connects with the base of the protrusible penis. The oviduct opens into the lumen of the mucus-gland. A region of convoluted tubules close to the point of the opening of the oviduct into the mucus-gland is the albumen-gland. The bursa copulatrix is a blind tubular invagination of the lateral body-wall which in mature specimens stores spermatozoa received at copulation. There are thus three external genital openings.

Copulation is almost invariably reciprocal, the penis of each individual being inserted into the bursa copulatrix of the other. Secretions from the glandular linings of the bursa and of the vas deferens render spermatozoa exchanged at copulation physiologically mature; they are active and capable of fertilizing oocytes.

At oviposition oocytes are expelled from the ovotestis and transported by cilia to the main hermaphrodite duct. They then travel (through the dense masses of spermatozoa in this duct) along a narrow longitudinal ciliated tract of lining epithelium; this phenomenon was clearly observed in vivisected individuals. At the hermaphrodite valve the oocytes are deflected into the oviduct and are carried by cilia into the mucus-gland. In the lumen of this organ they meet active 'foreign' spermatozoa which have made their way from the bursa copulatrix. The oocytes and spermatozoa are now conducted along a tortuous predetermined ciliary path through the albumen-gland (where the primary egg-cases are secreted and where fertilization occurs), and then through the peripheral tubules of the mucus-gland (where successive mucous coats are added). The completed egg-string is expelled through the external female aperture and forms a ribbon which is attached to the substratum by pressure of the parent's foot upon it. Active spermatozoa may be observed moving within the primary egg-cases for some days after oviposition.

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INTRODUCTION

ALTHOUGH the gross morphology of the reproductive organs of a number of species of nudibranchs is known from scattered systematic writings by many authors, investigations into the microscopic structure and functioning of the various regions of the system are few. The most important studies are those of Chambers (1934) on *Embletonia fuscata*, Lloyd (1952) on *Archidoris pseudoargus* and other opisthobranchs, and MacGowan and Pratt (1954) on *A. montereyensis*. Chambers summarizes work done before 1934. The present paper is concerned with the reproductive mechanism of one of the most primitive living nudibranchs.

METHODS

Excised pieces of the genital tract were examined alive, often after intravital staining with neutral red. Material for sectioning was fixed in the fluids of Zenker (without acetic acid), Perényi, Bouin (made up with sea-water), or Lewitsky-saline (Baker, 1958). Of these, the last gave by far the best results for all types of tissue. The embedding medium employed was Hance's rubber wax (Gurr). Sections were cut at from 4 to 10 μ. Stains used included the Azan and iron haematoxylin of Heidenhain, and Mayer's haemalum counterstained with eosin and alcian blue 8 GS (Steedman, 1950).

THE LIFE-CYCLE AND THE STRUCTURE OF THE OVOTESTIS

Fig. 1 is a diagrammatic representation of observations made on samples of *Tritonia hombergi* Cuvier dredged at intervals through the year. The area from which the samples were taken was the *Modiolus modiolus* bed off the south coast of the Isle of Man (see Jones, 1951, p. 137). The picture which emerges is not as clear as might be desired, because the weather sometimes made it impossible to procure regular samples. Nevertheless, there can be little doubt that these observations show *T. hombergi* to have an annual life-cycle similar to those found in some dorid nudibranchs (Thompson, 1957, 1958*a*). Study of sections of the ovotestis of individuals taken from the samples removes any possibility of an alternative explanation; gonad development proceeds through the year in one direction, that leading towards sexual maturity; there are none of the resting or regression stages characteristic of perennial sexual cycles.

Fig. 2, A–E shows the structure of the ovotestis in specimens killed at various times of the year. There is little point in describing in any detail gonad maturation in *Tritonia*, for the essential features are known in many gastropods and *T. hombergi* does not differ from the usual pattern. Franzén (1955) describes stages in spermatogenesis of *Tritonia*. Much of his description was confirmed in the course of the present investigation, the only item eluding confirmation being the 'spiral keel' (Franzén, 1955, p. 426, *sk*) running down the sperm tail.

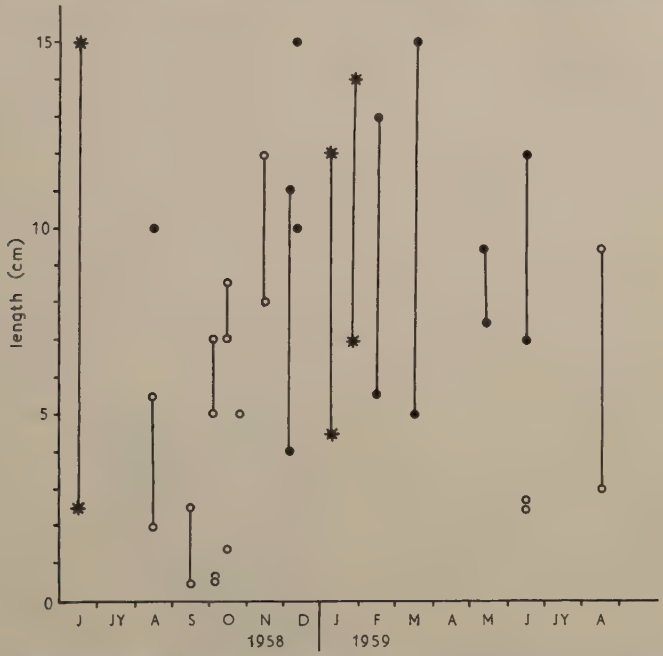

Fig. 1. Diagrammatic representation of collecting data in 1958 and 1959. The area from which samples were taken is described in the text. Maximum and minimum extended lengths only are considered for each sample. Empty circles denote samples containing sexually immature specimens; black circles denote samples consisting of mature specimens which immediately spawned in the laboratory; asterisks denote samples consisting of mature specimens accompanied by spawn found in the field.

The ovotestis of a sexually mature specimen killed immediately after having deposited a spawn mass showed that mature oocytes remained in only a small number of the tubular follicles (fig. 2, D). Within a few days of spawning, a fresh batch of oocytes begins to enlarge by the accumulation of cytoplasmic yolk, and in due course they in turn are expelled as a spawn mass. The number of such cycles of oocyte production in the life of a single *Tritonia* is not known, but is certainly not less than three. Possibly a phenomenon similar

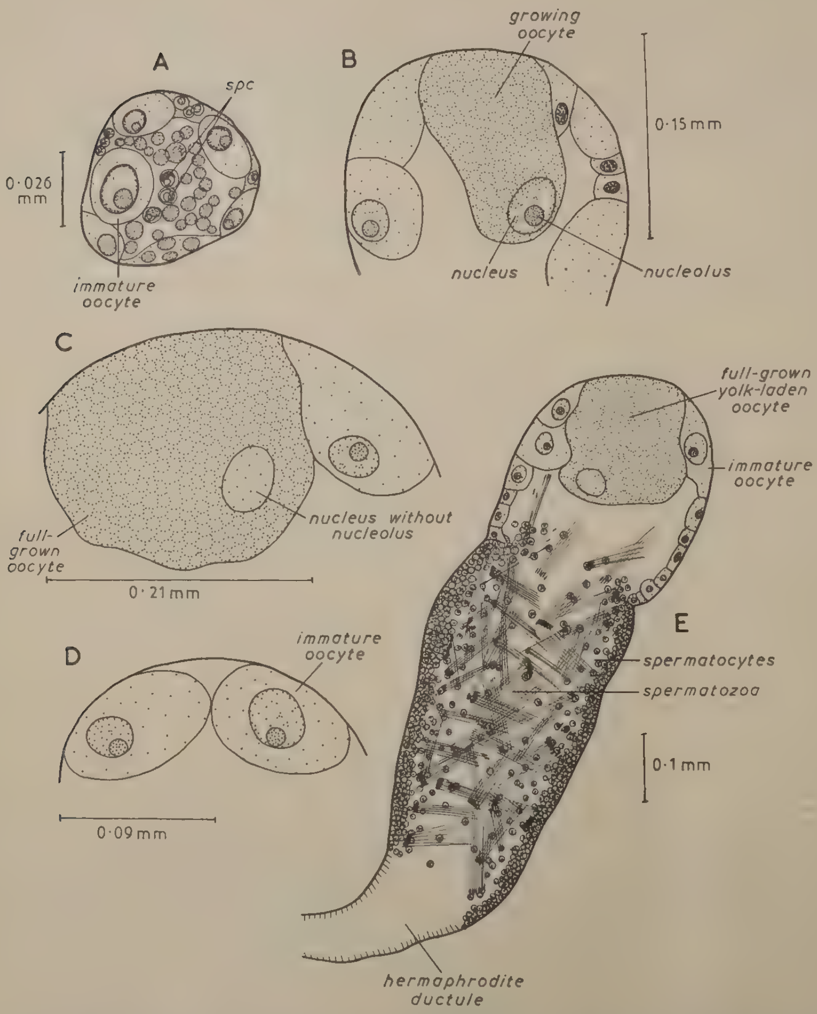

FIG. 2. Appearance of the ovotestis at various phases in the life-cycle. A, section through a single ovotestis tubule of an immature specimen collected on 26 Oct. 1958, showing immature oocytes and spermatocytes (immature oocytes have little or no cytoplasmic yolk and each of their nuclei contains a large nucleolus). B, portion of a section through an ovotestis tubule of an immature specimen collected on 17 Dec. 1958 (one of the oocytes shown in this plane is growing and accumulating cytoplasmic yolk). C, portion of a section through an ovotestis tubule of a mature specimen collected on 7 Jan. 1959, showing two oocytes, one full-grown, the other not (the nucleus of a mature oocyte lacks the nucleolus). D, portion of a section through an ovotestis tubule of a mature specimen collected in Jan. 1959 and killed immediately after spawning on 23 Feb. 1959 (only immature oocytes remain in the ovotestis). E, section through a whole ovotestis tubule of a mature specimen which had not spawned before death (February 1959).

to this provides an explanation for the enigmatic short-period cycles of spawn production in the dorid *Adalaria proxima* (Thompson, 1958*b*).

Spermatogenesis also continues throughout the period of sexual maturity, but the cycles of sperm production are not well marked and each of the genital tubules of the gonad of a single individual contains male gametes in all stages of formation (see fig. 2, E).

In the dorid *A. proxima* it was found (Thompson, 1958*a*) that, as the spawning season proceeds, the resources stored in the digestive gland are exhausted until a stage is reached when the organ is virtually shrunken out of existence; the adults die when this happens, even though the ovotestis may still contain numbers of apparently healthy oocytes and spermatozoa. Although some evidence points to the existence of a similar phenomenon in *T. hombergi*, no moribund post-sexual specimens were ever seen; it was, however, established by observation that the size of the digestive gland does decrease as the spawning season proceeds.

THE STRUCTURE OF THE MATURE REPRODUCTIVE COMPLEX

Ovotestis (fig. 2, E). This displays a rather primitive organization, the separation of the constituent tubules into male and female regions being rudimentary compared with other nudibranchs (except for some ascoglossans and the eolid *Coryphella*, Pelseneer, 1894). The ovotestis usually covers the whole external surface of the digestive gland. It consists of a large number of tubules, all orientated radially with regard to the centre of the digestive gland. Each tubule opens at its innermost extremity into a factor of a complex duct system (see fig. 2, E), lined by a simple ciliated epithelium and leading anteriorly to the hermaphrodite duct. In any individual tubule, the female region is distal to the male (fig. 2, E); in some tubules the female region may be divided into two or three lobules separated from the proximal part of the tubule by slight constrictions. A fine network of muscle-fibres lies around each tubule. At maturity, the tubules each contain a small number (about 4 to 6) of mature oocytes and a great number of morphologically mature spermatozoa. The former are attached to the wall of the tubule while the latter lie free in the lumen. The mature oocytes give a cream-yellow colour to the gonad in life; this colour may be seen through the foot of a live specimen.

Hermaphrodite duct (figs. 3; 4, C, D, E). The hermaphrodite duct is a simple tubular structure; there is no obvious division into male and female regions. The initial region after leaving the ovotestis is narrow and lined by a simple ciliated epithelium (fig. 4, E). Outside this epithelium is a layer consisting of bundles of circular and longitudinal muscle-fibres and outside this in turn a spongy layer consisting of blood-spaces.

The duct soon widens to form the vesicula seminalis (see figs. 3; 4, C); here huge numbers of spermatozoa collect in the lumen, which is bordered by an epithelium of complex aspect. A narrow region running longitudinally through the vesicula seminalis is ciliated, the remainder of the epithelium being composed of columnar, non-ciliated cells. The ciliated zone is clearly visible in preparations of live material. Outside the lining epithelium is a thick layer consisting of bundles of circular and longitudinal muscle-fibres and outside this in turn a layer of blood-spaces. Live spermatozoa in squash preparations from the ovotestis and vesicula seminalis appear mature in all but one respect: they are, except a few sluggishly moving individual spermatozoa, immobile and lacking any common orientation. Since both these qualities are indicative of physiological (as distinct from gross morphological) maturity in gastropod spermatozoa, it is clear that there is no possibility of self-fertilization occurring in the hermaphrodite tubules of the ovotestis. This is borne out by other observations.

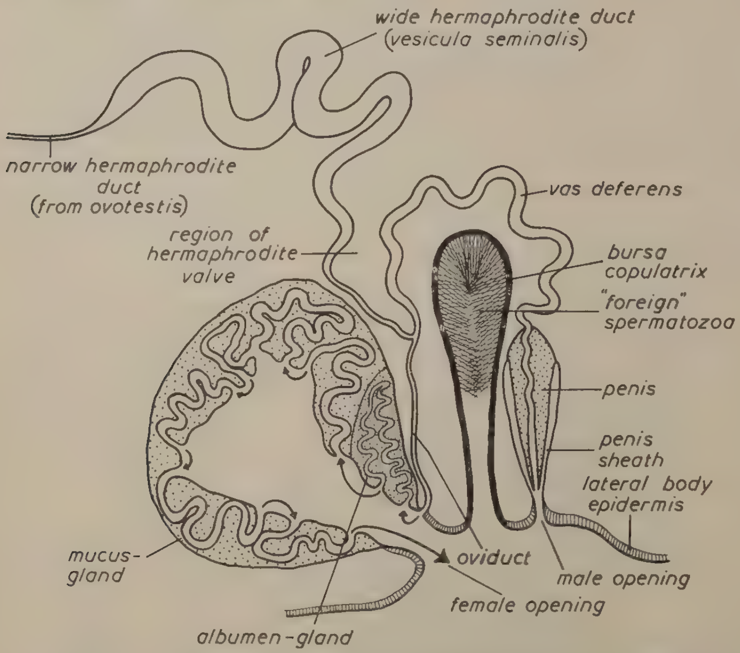

FIG. 3. Diagram showing the relations between the various parts of the anterior genital mass in a mature individual. The proportions are somewhat distorted in the interests of clarity. The arrows show the approximate path followed by oocytes entering the mucus-gland from the oviduct during the spawning process. Anterior is to the right of the page.

FIG. 4 (plate). Photomicrographs of sections (stained with Azan) through regions of the adult reproductive tract.

A, bursa copulatrix after copulation.
B, portion of the same, at greater magnification.
C, wide region of the hermaphrodite duct (vesicula seminalis).
D, portion of the same, at greater magnification.
E, narrow region of the hermaphrodite duct.
F, vas deferens.

bl, blood spaces; *cil*, ciliated region of lining epithelium; *gl*, glandular lining epithelium; *m*, muscle layer; *non-cil*, non-ciliated lining epithelium; *non-or*, non-orientated spermatozoa *or*, orientated 'foreign' spermatozoa.

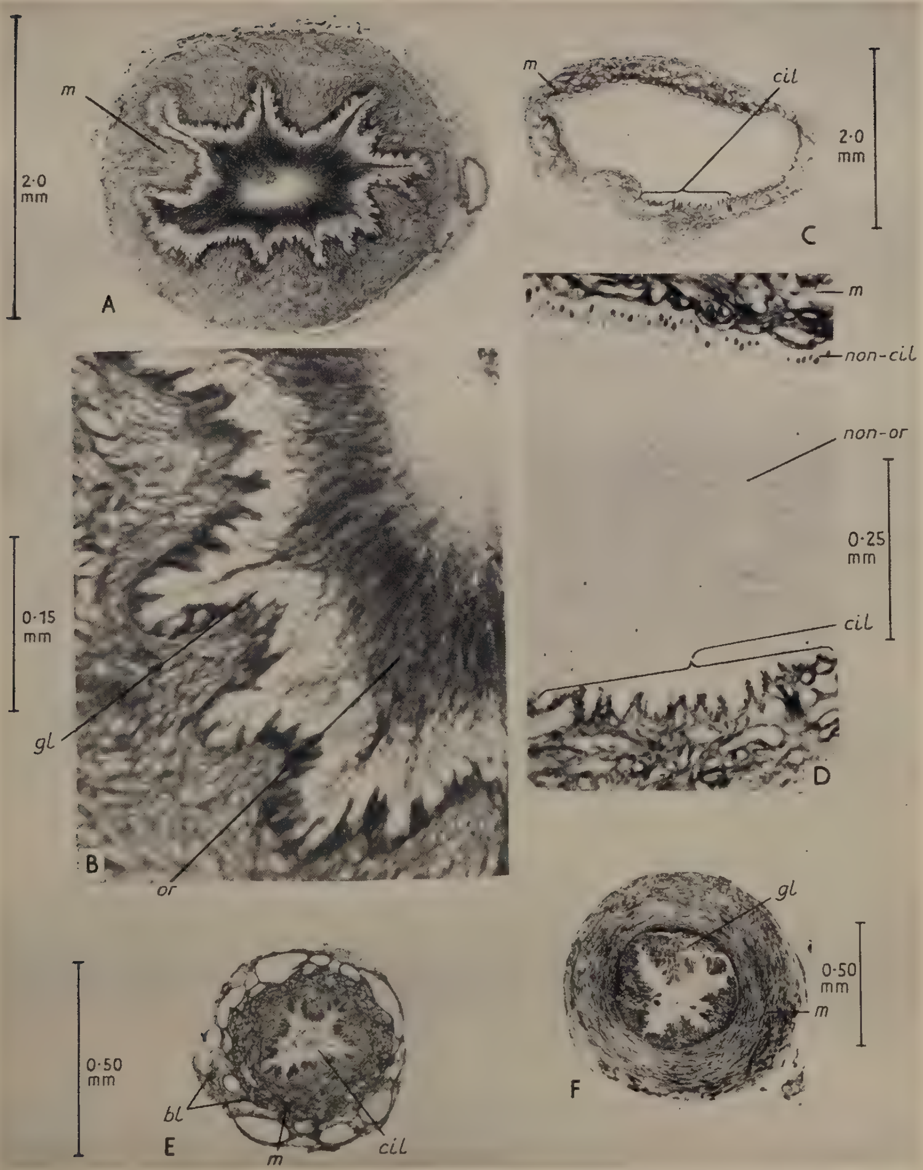

FIG. 4

T. E. THOMPSON

The vesicula seminalis, which appears grey in life owing to the colour of the spermatozoa within, lies in a few loose coils, bound together by connective tissue, dorsal to the anterior genital mass (fig. 8). At its anterior extremity it narrows and appears to enter the mucus-gland; actually it runs along a furrow between the two lateral halves of the gland. Deep inside the body of the mucus-gland the hermaphrodite duct divides into two. One of these further ducts is the oviduct, the other the vas deferens: it is at this bifurcation that the separation (such as it is) of the male from the female genital products occurs. The bifurcation may be best visualized as a straight tube (one end of which is the vas deferens, the other the oviduct) into the lumen of which projects a small papilla, through which opens the hermaphrodite duct (fig. 5). The whole region is closely surrounded by muscle-fibres and aggregations of nerve-cells. The mode of functioning of this 'hermaphrodite valve' clearly involves the contraction of regions of this musculature, the degree of co-ordination required being reflected by the unusual aggregation of nervous elements near by. A valve of this type was described for *Archidoris montereyensis* by MacGowan and Pratt (1954), but it is quite unlike that which Chambers (1934) found in *Embletonia fuscata.*

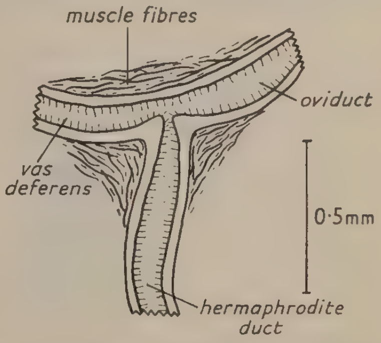

FIG. 5. Diagrammatic reconstruction of the region of bifurcation of the hermaphrodite duct to form oviduct and vas deferens.

In *Tritonia* this valve appears efficiently to prevent the entry of oocytes into the vas deferens during the spawning process, for female gametes were never found in the male regions of the system. However, the valve by no means prevents spermatozoa from entering the oviduct from the vesicula seminalis, although they do not do this in any quantity. Spermatozoa which have obviously passed through the valve into the female parts of the tract may be found in any part of the oviduct. Duncan (1958) calls attention to a similar phenomenon in the pulmonate *Physa fontinalis.* These 'escaped' sperms are not physiologically mature and almost certainly cannot result in normal circumstances in self-fertilization.

Vas deferens (figs. 3; 4, F). From the point where the vas deferens branches off from the hermaphrodite duct (fig. 3), it widens into a thick, muscular duct, leading after a few coils to the inner extremity of the penis. The lining epithelium is columnar and the cells are loaded with secretory globules of various sizes and staining qualities; this secretion may be termed, to facilitate comparison with other gastropods, the prostatic secretion. These columnar cells are ciliated. Outside the epithelium is a thick zone of circular and longitudinal muscle-fibres, lying in bundles in a connective-tissue matrix. In life the vas deferens appears white.

Penis (fig. 6). The penis is a large organ lying in an ectodermal sheath.

Distension of the connective tissue surrounding the final region of the vas deferens is responsible for the erection and eversion of the penis. When fully extended (fig. 6, B), the penis is a translucent white organ, bearing on its anterior face a small, keel-like structure; this keel is concerned with guiding the penis into the bursa copulatrix of a copulating partner. The whole organ is mobile and the tip is capable of considerable movement.

Extrusion of the penis is by no means invariably a prelude to copulation but is a frequent sign of impending death in an unhealthy specimen.

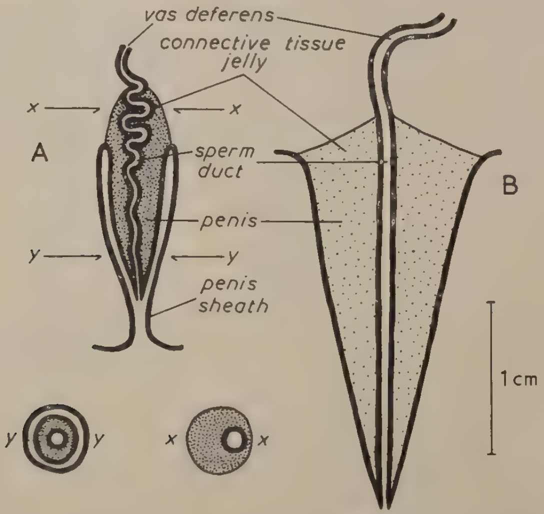

FIG. 6. Diagrams of the penis. A, sagittal section through the penis and its sheath in the resting (retracted) condition (sections in the planes marked on this figure are shown below it). B, sagittal section through the penis when distended and extruded.

Oviduct (fig. 3). After leaving the hermaphrodite valve the oviduct lies in the furrow separating the two lateral halves of the mucus-gland; it is a simple, non-glandular tube, lined by a ciliated epithelium. It opens into the lumen of the mucus-gland, close to the external female genital opening.

Albumen-gland (figs. 3; 7, A). This small organ appears pale pink in life and consists of a coiled tube, held to the outside of the mucus-gland by connective tissue, and lined by a glandular epithelium. The secretory cells are columnar and are separated by narrow ciliated cells. The tubular gland opens at both its extremities into the main lumen of the mucus-gland, close to the entry of the oviduct (fig. 3).

Mucus-gland (figs. 3; 7, B, C). The mucus-gland is a large organ, pale cream to white in life; the outlines of many of the convoluted tubules of which it is composed may be seen with the naked eye in dissections (fig. 8). The gland consists of a broad central lumen (lined by a low non-glandular ciliated epithelium), into and out of which open numerous convoluted tubules. These tubules are lined by columnar secretory cells and narrow ciliated cells. The secretory cells are of two kinds (fig. 7, B, C); regions consisting of one type lead abruptly into regions lined by the other without any apparent governing rule. The main central lumen of the gland takes the form of a V-shaped space in sections through the organ and this lumen leads down to the external female aperture. Close to this external opening are the openings of the oviduct (one) and the albumen-gland (two) (fig. 3). The final portion of the female system is clothed with cilia, all of which serve to convey particles out of the tract.

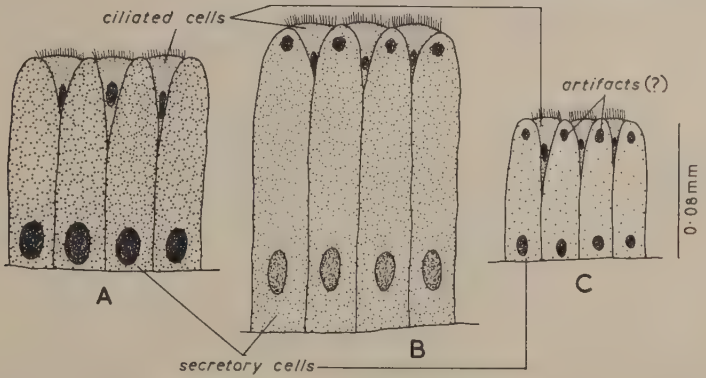

FIG. 7. Glandular cells of the albumen- and mucus-glands. A, portion of a section through the lining of the albumen-gland. B, portion of a section through the lining of the mucus-gland. C, the same, showing a second type of secretory cell.

Bursa copulatrix (figs. 3; 4, A, B). The bursa is a blind, tubular, ectodermal invagination, opening to the exterior through a ciliated duct close to the oviducal aperture. It has no open connexion with any other part of the reproductive system. In mature specimens the bursa appears pink to pale purple owing to the presence of a pigment in the wall. The organ serves for the reception and storage of 'foreign' spermatozoa received during copulation.

The lining epithelium of the bursa consists in sections of a peculiar 'bubbling' layer of cells. These cells are columnar and each contains a huge vacuole; the bursting of numbers of these vacuoles gives a hazy appearance to the adjacent lumen. This epithelium is not ciliated.

In individuals which have copulated the bursa is distended with greyish masses of spermatozoa all of which are orientated with their heads facing (but not embedded in) the wall (fig. 4, A, B). These sperms are active and it is inferred (in view of the fact that spermatozoa from the vesicula seminalis are immobile) that this motility was instigated by secretions from the prostate or from the bursa or from both.

An interesting phenomenon is the apparent synchrony of sperm-tail movement in sperms which face the same way. Sections show that the waves of action passing along the sperm-tails (which presumably all ceased at the precise moment when the fixative entered and they were killed) are in the same state in all the sperms visible in any section. This may give a striated appearance to masses of sperms in histological preparations (fig. 4, B).

Physiologically mature spermatozoa. Motile sperms of *Tritonia* progress by spiral waves passing backwards along the sperm-tail. Each sperm measures approximately 0·28 mm in length and approximately 20 waves may be present along the tail at any one time. Spermatozoa from the vesicula seminalis or from the ovotestis also show the tails to be spiral, but in this case the spiral is stationary and the sperms immobile.

External genital apertures (fig. 3). There are three of these. From anterior to posterior they are the external male opening (that of the penis or the penis sheath), the female opening (that of the mucus-gland), and the opening of the bursa copulatrix. These are capable of great dilation.

THE MODE OF FUNCTIONING OF THE REPRODUCTIVE ORGANS

Copulation. Copulation is almost invariably reciprocal, two individuals coming together head to tail, with their right sides apposed. On rare occasions pairs were found to consist of one 'passive' individual acting as a female alone, the other acting as a male. Individuals are promiscuous and copulation occurs numerous times, not always being followed directly by oviposition. Pairs may be found in copulation throughout the breeding season. Copulation may continue for many hours without the animals appearing to move.

The erection of the penis and its extrusion (fig. 6) are brought about through the uptake of fluid by the connective tissue surrounding the base of the organ. During copulation the penis of each partner is inserted into the bursa copulatrix of the other and spermatozoa are forced through the central duct of the penis by muscular contractions of the vesicula seminalis and the vas deferens.

Oviposition (figs. 8, 9). Oviposition or spawning occurs usually, but not always, during the hours of darkness (under laboratory conditions). The production of even a relatively short spawn ribbon takes many hours.

Spawn from the field had usually been attached at oviposition to colonies of *Alcyonium digitatum* (on which the adults feed) or to shells of *M. modiolus.* In the laboratory, however, the glass sides of culture vessels were almost invariably preferred to *Alcyonium* colonies and *Modiolus* shells. This peculiar phenomenon has also been noted for the dorid nudibranch *Adalaria proxima* (Thompson, 1958*a*).

The process by which oocytes are expelled from the tubules of the ovotestis has never been directly observed, but it seems probable that contraction of the muscle-fibres surrounding the tubules is the effective agent. This was also suggested by Chambers (1934) for *Embletonia.* In the ductules leading towards the main hermaphrodite duct, the oocytes are carried along by the lining cilia. They travel through masses of morphologically mature spermatozoa, but self-fertilization does not occur; there is ample reason for believing that these non-motile sperms are physiologically immature. While the oocytes are moving along the ducts their nuclei begin the final phases of maturation.

Large numbers of oocytes come to collect in the ductule system near the anterior extremity of the digestive gland, for the initial region of the hermaphrodite duct is so narrow as to permit the entry of only one oocyte at a time.

The oocyte cytoplasm at this stage is highly plastic, and when numbers are packed together their shapes are modified readily to allow a high degree of concentration. They do not possess any power of independent movement.

Oocytes are carried through the initial narrow region of the hermaphrodite duct by the action of the lining cilia (fig. 4, E). In the wide region of the duct (the vesicula seminalis) only a narrow longitudinal strip of the lining is ciliated (fig. 4, C, D); this strip lies in a 'ventral' position all along the coiled duct, but, since the animal itself may be attached to a vertical surface while spawning, this is probably without significance. The longitudinal ciliated tract serves to conduct the oocytes through the masses of non-motile spermatozoa filling the vesicula seminalis. If a spawning specimen is vivisected, the stream of oocytes passing through the hermaphrodite duct may be observed (fig. 8). To see this, in a dissection from the dorsal aspect, part of the duct must be lifted and turned over. The stream of oocytes is not held to the longitudinal ciliated tract simply by the influence of gravity. The speed of their passage along the tract was between 2 and 3 mm/sec; some oocytes pass along in clumps, others singly. No peristaltic movements of the wall of the vesicula seminalis were observed to occur, and it is certain that the movement of oocytes through this region of the system is brought about solely by ciliary action.

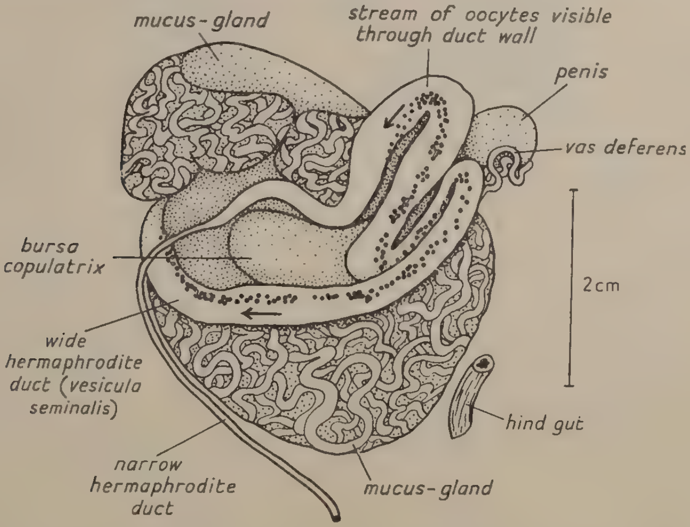
mucus-gland
stream of oocytes visible through duct wall
penis
vas deferens
2 cm
bursa copulatrix
wide hermaphrodite duct (vesicula seminalis)
hind gut
narrow hermaphrodite duct
mucus-gland

FIG. 8. The anterior genital mass of a vivisected specimen during the spawning process, from the dorsal aspect. Part of the vesicula seminalis has been reflected to show its under-side. The arrows show the direction in which the oocytes can be seen (through the translucent wall of the vesicula seminalis) to travel. Anterior is to the top of the page.

At the bifurcation of the hermaphrodite duct (figs. 3, 5) the oocytes pass into the oviduct; none was ever found in the vas deferens. A number of non-motile sperms enter the oviduct along with the oocytes, but play no part in the fertilization process, as far as could be ascertained. The oviduct is ciliated throughout.

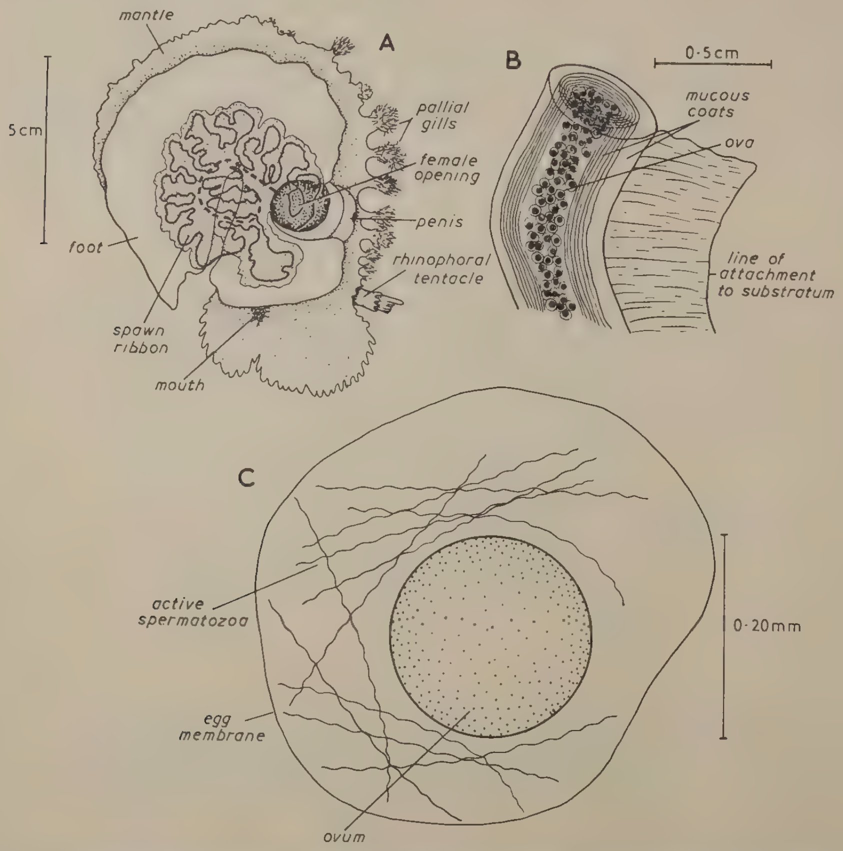

FIG. 9. The spawning process. A, adult in the act of spawning, viewed through the under-side of a glass plate (the line of attachment of the spawn ribbon to the glass substratum is shown by an interrupted line). B, portion of a completed spawn ribbon. C, single ovum, enclosed in its primary egg-membrane.

The oviduct opens into the main lumen of the mucus-gland, near the external female aperture (fig. 3). The oocytes are immediately taken up by ciliary currents and impelled into the tubular albumen-gland, together with a stream of mature, motile spermatozoa from the nearby bursa copulatrix. The means by which these sperms travel the few millimetres from the bursa to the mucus-gland lumen is not certain; ciliary currents in the relevant areas are in the contrary direction, and a contraction of the musculature of the bursa would appear to be most likely to result merely in a loss of sperms to the exterior. The most probable explanation is that the spermatozoa travel from the opening of the bursa up into the lumen of the mucus-gland by their own efforts. Analogous phenomena are well known in other groups of animals.

A mixture of mature spermatozoa and oocytes enters the ciliated lumen of the albumen-gland; fertilization occurs, and the spherical shape of the fertilized ovum is assumed. Secretions from the columnar epithelium of the albumen-gland form the primary egg-cases, which are ovoid. In some instances a single primary case may enclose more than one ovum. Sperms are usually enclosed within the egg-cases, the number so trapped varying from egg to egg and from adult to adult. After having traversed the coiled albumen-gland, the ova, each enclosed in a single case, are again passed into the main lumen of the mucus-gland.

In the mucus-gland ciliary action assembles the ova into a string which is then conducted on a complex, winding journey culminating in the expulsion of a ribbon-like egg mass from the external female genital opening (fig. 9, A). The string of ova passes into and out of the numerous tubular diverticula leading from the main lumen of the mucus-gland; in these tubules the protective mucous coats of the spawn mass are added, and the string travels slowly along its path, which is predetermined by ciliary tracts. As the completed egg string emerges from the greatly dilated female genital aperture, the pressure of the foot upon it causes the free edge to become attached to the substratum (fig. 9, A). This can be demonstrated in a live spawning specimen; after a few minutes it becomes impossible to detach the ribbon from its substratum without causing damage to it. As more and more of the spawn is extruded through the genital opening, the animal moves in a slow spiral so that the completed ribbon has this form (fig. 9, A).

THE STRUCTURE OF THE EGG MASS

A portion of a spawn mass is shown in fig. 9, B; fig. 9, C shows a single ovum with its membrane. Spermatozoa remain visible within the egg-cases (fig. 9, C) for up to a fortnight after oviposition, but then are no longer seen; they lose their motility within a few days after oviposition.

The maximum range of variation in the size of the ova of *T. hombergi* was from 0·19 to 0·21 mm in diameter; these figures were obtained by examination of ova from a large number of spawn masses. The number of ova in a single spawn mass is also greatly variable. A single example will suffice to show how many were encountered: a ribbon which measured 42 cm when uncoiled (but without any attempt to correct any of the minor kinks) contained an average number of 1,236 eggs per cm. Thus the total number of eggs in the ribbon was approximately 52,000. The parent individual in this case was 14 cm in length.

The relative abundance of twin, triplet, and quadruplet ova varied considerably from one mass to another; my observations may be loosely summarized by stating that twins were often seen, triplets rarely, and quadruplets only very rarely. There are always a number of empty egg-cases at each end of a spawn ribbon, confirming that the primary membrane is not a true fertilization membrane. (The egg-membranes are secreted by the albumen-gland.)

This work was begun during my tenure of a Leverhulme Fellowship in the University of Liverpool and completed at University College, Cardiff. I am deeply grateful to Mr. J. S. Colman and to Professor James Brough for their critical reading of the manuscript and for the provision of laboratory facilities. In addition it is a pleasure to record my indebtedness to Mr. R. G. Hartnoll, Mr. P. J. Miller, and Dr. K. Reddiah, for their help in collecting the material on which this paper is based. Dr. H. H. Williams and Mr. W. O'Grady gave advice and help in taking the photomicrographs.

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A Note on the Habits and Nutrition of *Solemya parkinsoni* (Protobranchia: Bivalvia)

By G. OWEN

(*From the Department of Zoology, University of Glasgow*)

With two plates (figs. 1 and 2)

SUMMARY

Adult specimens of *Solemya parkinsoni* Smith, embedded in mud at a depth of 50 cm or more, were collected near low water (spring tide). The animal burrows with the anterior end downwards and does not maintain an opening to the surface. An inhalant current is drawn into the mantle cavity anteriorly on each side of the foot, while an exhalant current leaves by the single, posteriorly situated aperture. This is probably a respiratory current, bottom material entering the mantle cavity as a result of the muscular activity of the mantle and foot.

The course of the alimentary canal is described, and the problem of feeding and nutrition correlated with the extreme reduction of the gut exhibited by *S. parkinsoni* discussed. It is suggested that an initial breakdown of organic material may take place in the mantle cavity.

INTRODUCTION

LIVING specimens of *Solemya parkinsoni* Smith were collected during a brief visit to the Marine Biological Station, Portobello, New Zealand. The intention was to investigate the gut and to compare the results with those obtained from similar studies on members of the Nuculidae (Owen, 1956) and Ledidae. This was not possible in the time available. Previous workers (Pelseneer, 1891; Stempell, 1899; Yonge, 1939) have remarked that the gut of *Solemya* is noteworthy for its small size but this does not prepare one for the extreme reduction of all parts of the gut exhibited by *S. parkinsoni*, particularly since the gut in the Nuculidae and Ledidae is rather large. Fig. 1, A, B represents cross-sections of corresponding regions of the stomach of *Nucula sulcata* (shell size, 2·0 × 1·5 cm) and *S. parkinsoni* (7·5 × 3·0 cm) and illustrate the exceptionally small size of the gut in the latter. This is further emphasized by the fact that a first attempt to expose the stomach and intestine failed, the anterior aorta where it passes through the gonadial tissue being dissected by mistake (see fig. 3); the intestine in this region is less than half the diameter of the aorta. It is therefore pertinent to inquire what part the reduced gut plays in the nutrition of a rather bulky and potentially active animal. Owing to the difficulty of obtaining living specimens, the enigma presented by the gut of *S. parkinsoni* was not solved, but it is thought worth while to give a brief account of observations on preserved material. Observations on the habits of the living animal made during the brief visit to Portobello are also included, since previous accounts of the mode of burrowing and the position of the inhalant current vary.

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OBSERVATIONS ON THE LIVING ANIMAL

Observations on the habits of *Solemya* have been made by Drew (1900), Morse (1913), and Yonge (1939). According to Morse, specimens of *S. velum* and *S. borealis* frequently burrow with the anterior end downwards, while Drew (1900) reported that '*Solemya* lives in rather hard mud, and, I think, keeps its burrow more or less open'. Yonge (1939), on the contrary, found that *S. togata* always burrowed with the anterior end downwards, and on no occasion was there any indication of an opening to the surface. Specimens of *S. parkinsoni* embedded in sandy mud at a depth of 50 cm or more were obtained near low water (spring tide); a few small specimens were found nearer the surface. The surface of the substrate was searched carefully for any indication of the presence of *S. parkinsoni*, but without success, and specimens were obtained by random digging in the area. Attempts were also made to determine the orientation of the animal within the substrate. A number of specimens were obtained with the foot cut off, but the remainder of the animal undamaged. On these occasions, the animal was invariably thrown out on to the discarded heap of mud while the severed portion of the foot remained in the substrate. This suggests that the animal lay either horizontally or vertically, but with the foot extending downwards. The latter would appear more likely and this is supported by other observations and by information subsequently received from Dr. Batham at Portobello.

Further observations on the burrowing activities of *S. parkinsoni* were made in the laboratory, and the sequence of burrowing is essentially similar to that described for *S. togata* by Yonge (1939). This is illustrated in fig. 1, C–E, where the ability of the terminal region of the foot to expand, and so obtain a purchase in the substrate, is clearly shown (see also fig. 2, D). Morse (1913), in support of his contention that *S. velum* frequently burrowed posterior end downwards, described how specimens placed on the sand with the ventral side uppermost protruded the foot ventrally and arched anteriorly to press against the substrate, thus lifting the anterior end of the shell and depressing the posterior end. Similar movements were observed in the laboratory with *S. parkinsoni* (fig. 2, D), but the posterior end of the animal was depressed only when the foot failed to penetrate the substrate sufficiently to provide the necessary purchase to draw the anterior end of the animal into the substrate. There seems little doubt that *S. parkinsoni* normally burrows obliquely into the substrate with the anterior end downwards (fig. 1, E) and, moreover, does not maintain an opening to the surface. Swimming of *Solemya*, described by Drew (1900), was observed on one occasion and is spectacular. As one observer commented, 'It exhibits the sort of speed that in molluscs one normally associates with cephalopods'. That swimming is a normal feature of the behaviour of *Solemya*, however, is unlikely (Yonge, 1939).

FIG. 1 (plate). A and B, sections of the stomachs of *N. sulcata* and *S. parkinsoni* respectively, photographed at the same magnification.

C–E, a specimen of *S. parkinsoni* burrowing into sandy mud.

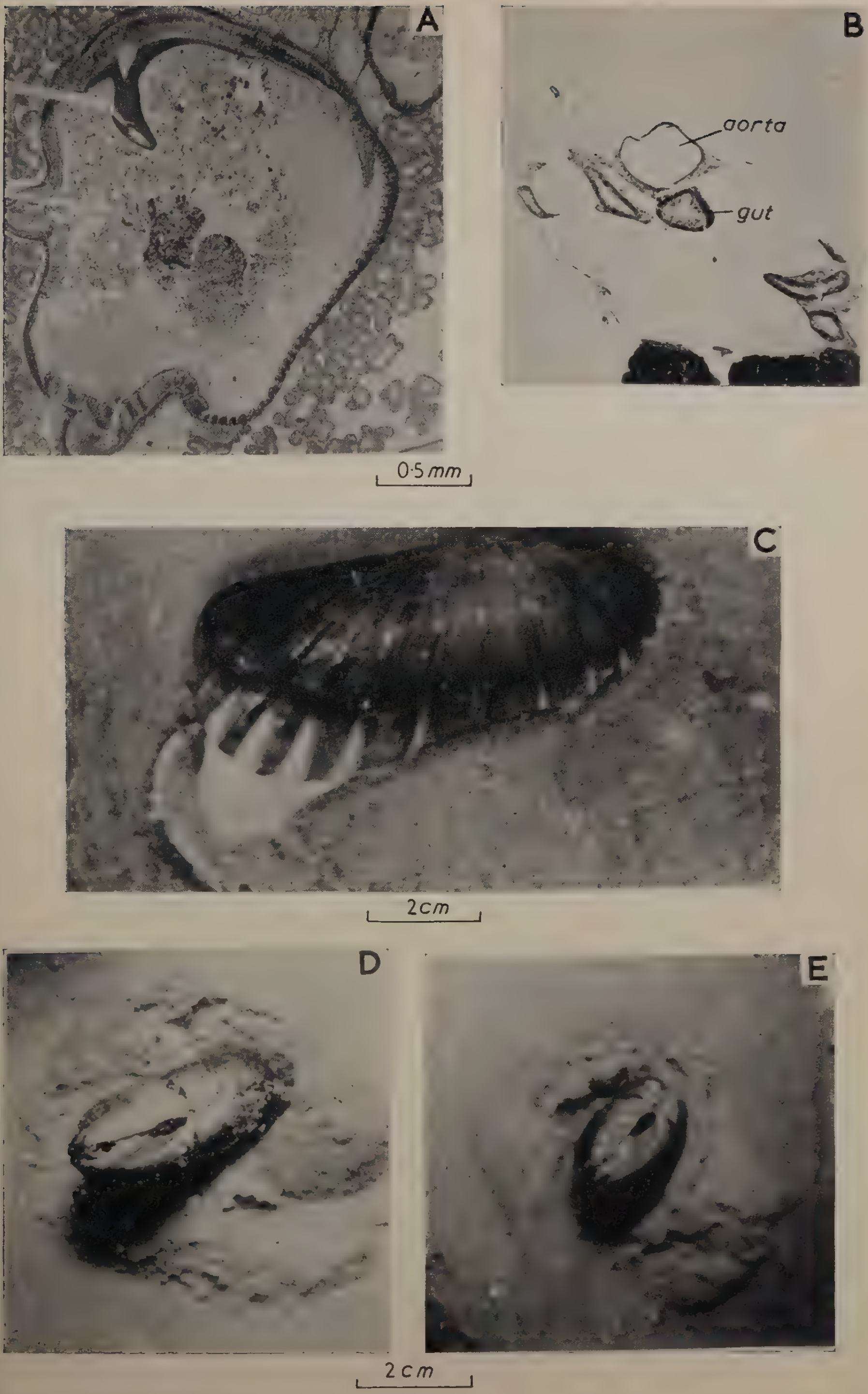

FIG. 1

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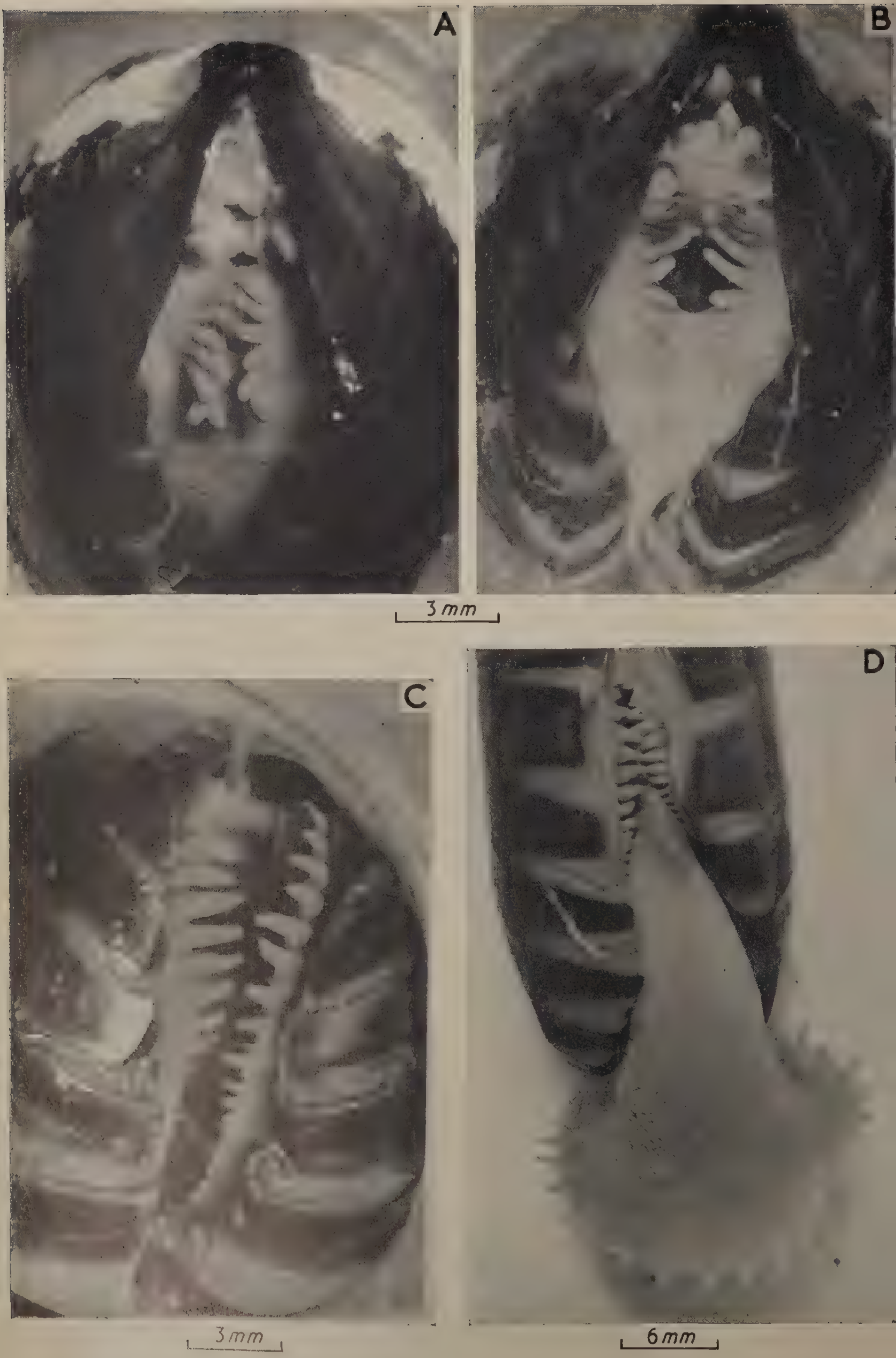

Fig. 2

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The single posterior aperture of *Solemya* received considerable attention from earlier workers, many of whom (Deshayes, 1845–8; Stempell, 1899; Morse, 1913) described it as an anal-branchial siphon. Both Kellog (1892) and Morse (1919) described changes in the form of the opening and noted how, on occasions, it is partly divided into dorsal and ventral regions, which, they suggested, may represent exhalant and inhalant apertures respectively. Orton (1913), on the contrary, states that the inhalant current is drawn in anteriorly, dorsally to the foot, while Yonge (1939), owing to the small size of the specimens of *S. togata* examined, found exact determination of the inhalant current difficult. He suggested that the arrangement of the gills and the shape and habits of *Solemya* all indicate the presence of an anterior inhalant current. The partial division of the posterior aperture was observed in *S. parkinsoni* (fig. 2, A, B), the lateral margins being drawn together so that the tentacles in this region interlock and divide the aperture into a small dorsal opening at the base of the dorsal series of tentacles and a larger ventral one. Nevertheless, an exhalant current only was observed in this region, sometimes issuing from both dorsal and ventral regions of the aperture, and on occasions from one portion only, the other being partially closed. An inhalant current is drawn in on each side of the foot through both the antero-dorsal and postero-ventral regions of the pedal gape (fig. 3) between the interlocking tentacles borne by the mantle margins of these regions (fig. 2, C, D). Thus in *Solemya*, as in *Nucula* (Yonge, 1939), water is taken into the mantle cavity anteriorly and from beneath the surface of the substratum.

THE ALIMENTARY CANAL

The gut of *S. parkinsoni* follows more or less the course described by Yonge (1939), although the relative reduction of all parts of the gut would appear more extreme than in the much smaller *S. togata*. A small tubular oesophagus passes posteriorly between the anterior retractor muscles of the foot (fig. 3). At the posterior margin of these muscles there is a slight dilation associated with the entry of the ducts, one on each side, from the digestive diverticula; this represents the stomach (fig. 4). The diverticula comprise a small number of distinct, elongated tubules arranged on each side of the stomach and having a brownish-green colour in fresh material; numerous rather stout muscle fibres are inserted on to the distal ends of the tubules. Posteriorly to the stomach, the gut is continued as a slender greenish thread which swings slightly to the left of the mid-line and extends postero-ventrally into the gonadial tissue which forms the bulk of the visceral mass (fig. 3). Near the posterior margin of the visceral mass, the intestine bends sharply to run dorsally before penetrating the wall of the visceral mass. It then extends

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FIG. 2 (plate). Photographs of a living specimen of *S. parkinsoni*.
A and B, the posterior aperture.
C, the antero-dorsal inhalant region of the pedal gape.
D, the foot extended with the terminal region expanded. The postero-ventral inhalant region of the pedal gape is shown.

posteriorly as the rectum along the floor of the ventricle and of the posterior aortic bulb before opening at the anus on the postero-ventral face of the posterior adductor muscle.

The oesophagus, like the rest of the gut, is lined by a well-developed ciliated epithelium which attains a height of 40 to 50 μ. The diameter of the oesophagus is approximately 180 μ, while the stomach immediately before the entry of the ducts from the digestive diverticula is triangular in outline and measures 300 by 250 μ; the adjacent aorta has a diameter of over 500 μ (fig. 1, B). Muscle-fibres, which form a rather thin layer round the oesophagus, are well developed in the region of the stomach and form a layer 30 μ thick. Despite the small size of the stomach in *S. parkinsoni*, all the essential features of the bivalve stomach would appear to be present. Immediately posterior to the laterally situated slit-like apertures of the ducts from the digestive diverticula, the lateral and dorsal walls of the stomach are covered by a cuticular structure, ∩-shaped in section, which can be removed intact from the stomach it is presumably homologous with the extensive cuticular region of the stomach in the Nuculidae (Owen, 1956). Sections of the stomach reveal the presence of a small caecum which curves slightly to the left and corresponds with the dorsal hood of other bivalves. It is not visible in dissections. This region of the stomach is followed by one possessing a typical style-sac ciliation with a shallow groove—the intestinal groove—extending the length of the right wall. The style sac tapers into the intestine, which has a diameter of approximately 150 μ, and is surrounded by a layer of circular muscle-fibres some 5 to 6 μ thick. The gut extends to the anus with little change.

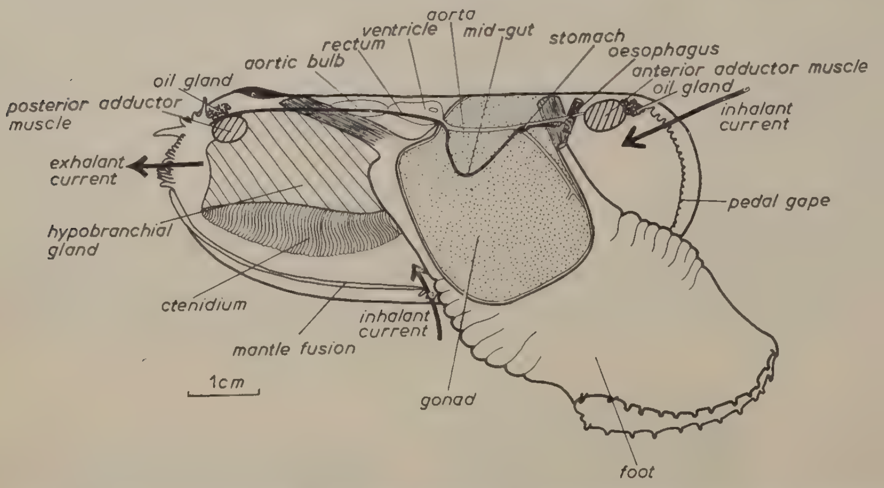

FIG. 3. The alimentary canal (solid black line) of *S. parkinsoni* viewed from the right side. Arrows indicate the inhalant and exhalant currents.

The digestive diverticula comprise a small number of finger-like tubules on each side of the stomach (fig. 4). The lumina of the proximal regions are lined by a ciliated epithelium which rests on a thick basement membrane underlain by well-developed muscle fibres. This region is similar to the ducts of the diverticula of other bivalves (Owen, 1955). Unfortunately, fixation of the distal regions of the tubules was poor, but cilia are undoubtedly present, while many of the cells contain numerous coloured inclusions.

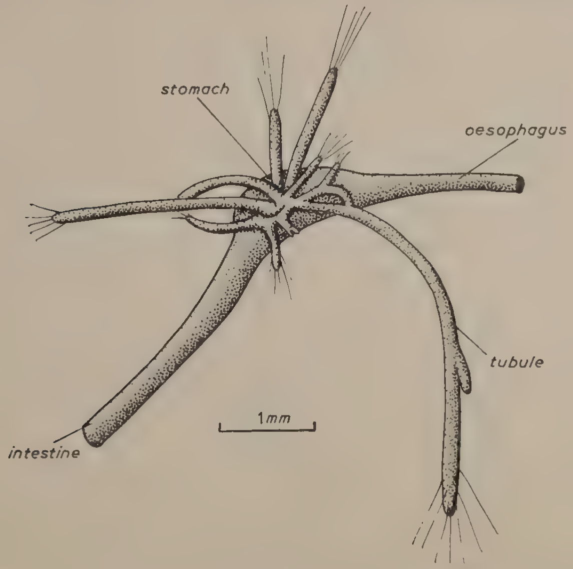

FIG. 4. The stomach and digestive diverticula of *S. parkinsoni* viewed from the right side.

DISCUSSION

Members of the three families of the Protobranchia—the Nuculidae, Ledidae, and Solemyidae—are deposit feeders. In the Nuculidae and Ledidae, the large palp proboscides extend between the valve margins and actively collect the bottom deposits. In the more specialized Ledidae, the palp proboscides are the sole food-collecting organs, while in the Nuculidae the part played by the small ctenidia is slight and probably incidental (Yonge, 1939). Selection of the collected material is exercised by the ridged surfaces of the palp lamellae, but in both families the stomach is large and invariably contains a high proportion of inorganic material, features characteristic of deposit feeders. In the Solemyidae the palp proboscides are smaller and it is the muscular activity of the mantle and possibly of the foot, correlated with the flexibility of the shell (Beedham & Owen, 1960), that results in large quantities of bottom material entering the mantle cavity (Yonge, 1939). According to Morse (1913), Orton (1913), and Yonge (1939), the sole food-collecting organs are the greatly enlarged ctenidia, and in this context the observations of Morse are of interest. 'The gills, however, are the food accumulators from which as we have seen the palp-appendages [palp proboscides] collect material for nutrition . . . the continual ingestion of food is indicated by the great quantity of excreta which is voided.' These observations do not suggest the extreme reduction of the gut exhibited by *S. parkinsoni*, and it is probable that Morse confused the mucus-bound material extruded from the mantle cavity (i.e. pseudofaeces) with true faecal material.

A more detailed analysis of the ciliary currents of the ctenidia and palps of *Solemya* has been presented by Yonge (1939). While Stempell (1899) suggested that the small size of the gut of *S. togata* was correlated with the high organic content of the substratum in which the animal lives, Yonge (1939) considered more significant the rigorous sorting of particles by the ctenidia and palps which results in only the finest particles entering the mouth. The frontal cilia of the ctenidial filaments convey fine particles to the inner margins of the ctenidia, where a weak anteriorly directed current conveys them towards the labial palps; large masses of material are conveyed posteriorly over the surface of the ctenidia and rejected. Ciliary currents over the inner and, in this case, smooth surfaces of the palp proboscides are directed into a narrow groove bounded by richly ciliated ridges representing reduced palp lamellae. Ciliary currents in the depth of the groove are directed towards the mouth while those over the ridges beat actively out of the groove and towards the rejection tracts of the foot. Thus according to Yonge only the very finest particles in the depth of the groove reach the mouth. Certainly, observations on the contents of the gut of freshly collected specimens of *S. parkinsoni* support the view that very little particulate material enters the gut, despite the presence of large quantities of bottom material in the mantle cavity. It is, therefore, difficult to appreciate how an animal which attains the bulk of *S. parkinsoni* can obtain sufficient nutriment by either intracellular, or for that matter, extracellular processes within the limits of such a reduced gut.

A possible explanation of the extreme reduction of the gut is suggested by observations on specimens of *S. parkinsoni* collected at Portobello, New Zealand. Two small specimens were left overnight in sea-water containing starch, iron saccharate, and titanium dioxide. They were fixed in Bouin's fluid and subsequently serially sectioned. Considerable quantities of starch, iron, and titanium dioxide were present in the mantle cavity but no trace of these substances was discovered either in the lumen of the gut or in any of the tissues. It is interesting to note, however, that the periodic acid/Schiff test and the iodine test revealed that the starch was undoubtedly undergoing a preliminary breakdown within the mantle cavity. While little value can be placed on the results obtained, and various explanations can be given for the observed breakdown of starch, there exists the possibility that digestion of organic material may take place in the mantle cavity of *Solemya*, and it is the products of this digestion that are ingested at the mouth. The ciliary currents of the ctenidia and palps would appear to prevent particulate material entering the gut. Morse (1913) has described the palp proboscides as long, semi-tubular structures with enlarged ends. Such structures, possessing the ciliation described by Yonge, would be admirably suited for collecting the products of any digestion which may occur in the anterior half of the mantle cavity; the ciliary currents of the reduced palp lamellae would serve to remove particulate material carried in the current created by the proboscides. The ctenidia could then be regarded as retaining, to a large extent, their primitive function of respiration. Their large size would be an adaptation to the specialized mode of life of *Solemya*, which lives buried in the substrate (40 to 60 cm deep in the case of *S. parkinsoni*) without direct contact with the water above.

There remains the question of the source of any enzymes which may be present in the mantle cavity. The characteristic glands present in the epithelium of the foot are worthy of further investigation in this respect. They are unlike any seen elsewhere in the Bivalvia and are present in both *S. togata* and *S. parkinsoni*, but are not found in members of the Nuculidae. It must be emphasized that the above hypothesis may be far removed from the true explanation of the extreme reduction of the gut exhibited by an animal which is an undoubted deposit feeder, and further investigation of the processes of feeding and digestion in the Solemyidae is desirable.

I am again indebted to Professor C. M. Yonge, C.B.E., F.R.S., for his advice and criticism and to Dr. Batham for permission to reproduce the photographs in fig. 1, C–E. I am indebted to Dr. Batham also for the facilities and hospitality offered me during a brief visit to the Marine Biological Station at Portobello, New Zealand. This visit was made while I was holding the John Murray Studentship of the Royal Society.

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On the Filamentous Elements in the Nucleoli of Chick Embryo Fibroblasts

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With one plate (fig. 1)

SUMMARY

Filamentous inclusions (= nucleolonemata) in the nucleoli of chick embryo fibroblasts and their behaviour during the cellular cycle are described. Their number is about the same as that of the macrochromosomes. They originate in anaphase as thin threads along the chromosomes. During the reconstitution of the daughter nuclei they aggregate within two nucleoli. They disintegrate with the prophase of the next mitosis.

INTRODUCTION

FILAMENTOUS inclusions have been found in the nucleoli of a variety of cells, with the light microscope (Estable & Sotelo, 1951, 1952; Lettré & Siebs, 1954; Denues & Mottram, 1955; Horstmann & Knoop, 1957) and with the electron microscope (Borysko & Bang, 1951; Bernhard & others, 1952 *a*, *b*; Porter, 1954; Yasuzumi & others, 1958). Because of their apparently different dimensions and also because of the different techniques applied, it has been questioned whether all these structures are homologous (Stich, 1956). In some instances no nucleolar inclusions were found at all (Rodkiewicz, 1959). It has been suggested (Serra, 1958, Rodkiewicz, 1959) that the filamentous inclusions found in nucleoli might be artefacts, caused by fixation or by the heterogeneous impregnation of a homogeneous ground substance. Unfortunately, even where these elements have been demonstrated during life, artifacts might have been caused by the methods employed (Hughes, 1952; Denues & Mottram, 1955).

A filamentous structure has been found with the electron microscope in chicken fibroblasts (Borysko & Bang, 1951). However, no individual filaments could be distinguished. Hughes (1952) found that the nucleoli of chicken fibroblasts can disintegrate into smaller fragments, which might or might not have existed during life as preformed elements. Lettré and Siebs (1954) claimed that the filaments found in the nucleoli of chicken fibroblasts are parts of chromosomes and that they give a positive Feulgen reaction.

Since the existence of filamentous nucleolar inclusions and their behaviour during the cellular cycle might be of great theoretical interest (Vincent, 1955), we have examined fibroblasts from chicken embryos with regard to these elements and to their formation after mitosis.

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METHODS

The cells were grown in hanging drop cultures, containing equal volumes of 25% chick embryo extract and chicken plasma. Vigorously growing cultures were fixed in 10% formaldehyde which had been neutralized with calcium carbonate. After thorough rinsing they were impregnated with silver nitrate according to a method of Cajal (Romeis, 1928). The impregnated preparations were examined under phase contrast.

RESULTS AND DISCUSSION

At the periphery of the cultures, the cells thinned out to such an extent that even the nucleoli were somewhat flattened, and the nucleolar inclusions, impregnated with silver nitrate, could be distinguished. In small, globular nucleoli they had the shape of granules. In nucleoli that were flattened the inclusions appeared mostly as nodular filaments (fig. 1, A, B). Since their thickness approaches the limit of resolution of the light microscope, it was not always possible to identify each single filament within a group with certainty, and their exact number could not be determined. Table 1 shows numbers of filaments and granules counted in several nuclei at the periphery of the culture. Two counts are given for each nucleus. The low count was derived by interpreting any group of nucleolar inclusions which was not clearly resolved in favour of the lower number, and the higher count was obtained by doing the opposite. It is worth noting that the numbers counted are about the same as that of the macrochromosomes (12, according to Brink (1959)).

TABLE 1

Number of nucleoli per nucleus	*Predominant shape of the nucleolar inclusions*	*Number of nucleolar inclusions*	
		low estimate	*high estimate*
2	granular	9	15
2	filamentous	14	18
2	filamentous	12	17
2	filamentous	10	13
2	granular	9	17
2	filamentous	12	15
2	filamentous	10	18
2	filamentous	12	13
2	filamentous	9	16
1	filamentous	15	19

We found no evidence that the nucleolonemata persist through mitosis. They disintegrate during prophase. In anaphase (fig. 2, A), a thin line appears along each chromosome. These lines are impregnated in the same way as the

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FIG. 1 (plate). A, nucleolonemata in a flattened nucleolus.
B, drawing of the same nucleolus, to show the position and the number of the filaments.

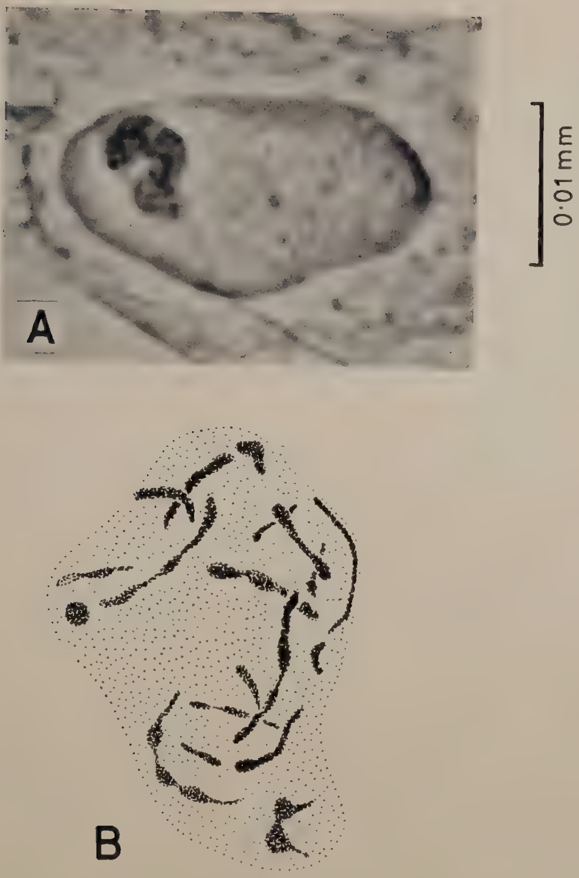

FIG. 1

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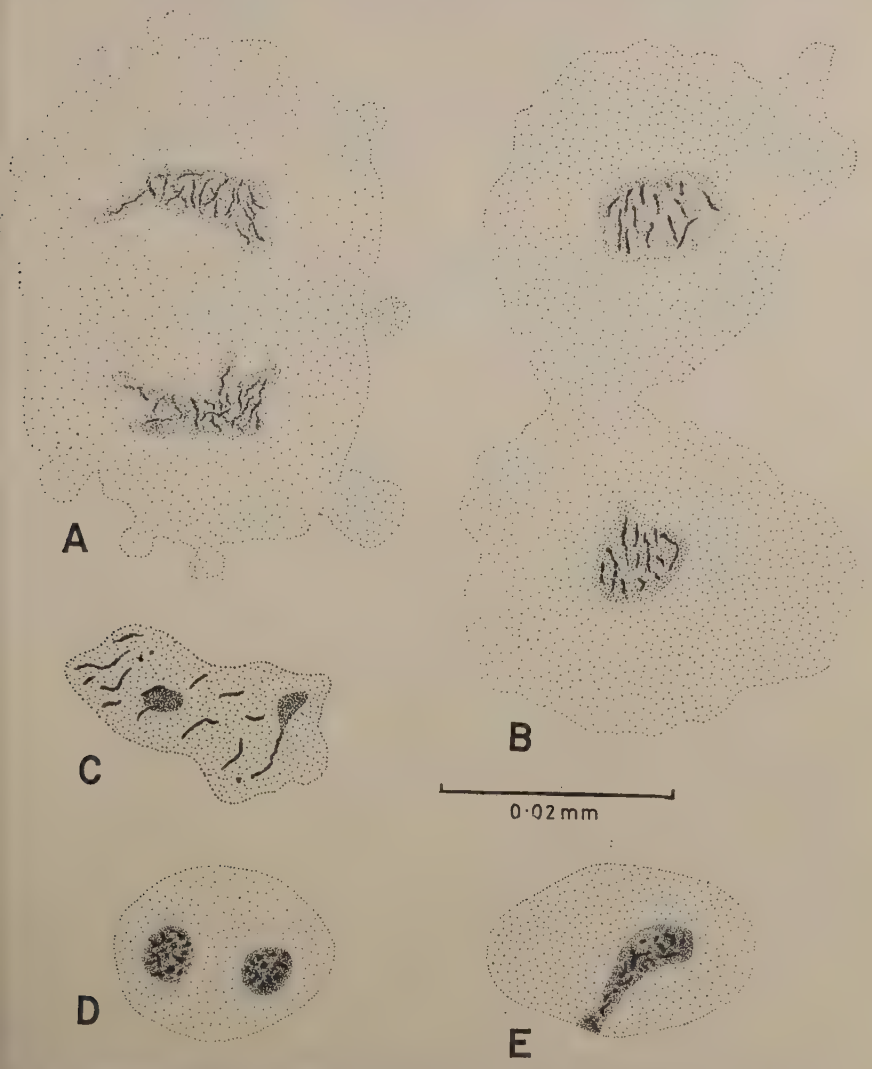
ucleolar inclusions. From here on the transition to the nucleolar inclusions in he daughter nuclei is continuous. In the daughter nuclei (fig. 2 B), while he individual chromosomes can no longer be distinguished, these threads



IG. 2. A, anaphase; nucleolonemata reappearing along the chromosomes. B, daughter nuclei. , daughter nucleus. Two small nucleoli; most of the nucleolonemata still in the extra-ucleolar parts of the nucleus. D, E, nucleolonemata within two (D) and one (E) nucleolus.

horten and become thicker. The reappearing nucleoli (fig. 2, C) first contain nly a few inclusions imbedded in the *pars amorpha,* while most of the short ods and filaments are still in the extra-nucleolar parts of the nucleus. In the nal stage of the reconstitution of the nucleus, all the nucleolonemata are ggregated in either one or two nucleoli (fig. 2, D, E), and no similarly impregnated elements are found in the rest of the nucleus any more. We found no evidence that any inclusion of the nucleoli gave a positive Feulgen reaction.

The continuous transition from the impregnated lines along the chromosomes, first to the short filaments and granules within the daughter nuclei, and then to an intermediate stage where some of the elements are found outside and some inside the nucleoli, until finally they are only found within the nucleoli, does not favour the argument that they are mere artefacts produced by fixation or heterogeneous impregnation of a homogeneous ground substance.

It is conceivable that the nucleolonema is produced by the dissociation of the chromosomes at anaphase into two complementary halves of which the one, containing the DNA, is to be transmitted through the generations, while the other has only a temporary existence through the next interphase.

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The Histology of the Neurosecretory System of the Adult Female Desert Locust, *Schistocerca gregaria*

By K. C. HIGHNAM

(*From the Department of Zoology, the University of Sheffield*)

With one plate (fig. 1)

SUMMARY

The pars intercerebralis of the brain of the desert locust contains about 2,400 cells n two groups, which stain with chrome-haematoxylin-phloxin and with paraldehyde-uchsin. On the basis of differences in size and staining reactions, four types of cell, alled A-, B-, C-, and D-cells may be differentiated. The A- and B-cells produce ifferent kinds of material; they are not thought to be stages in a secretory cycle. The C- and D-cells are probably not neurosecretory. The corpora cardiaca are ivided into two regions. One part stores neurosecretory material from the pars ntercerebralis and the other is glandular in appearance. Material discharged from he A- and B-cells in the immature female passes along the nervi corporis cardiaci I o the anterior parts of the corpora cardiaca. The mature female is characterized by he presence of very much larger amounts of material in the corpora cardiaca, in the ervi corporis cardiaci I, and in the A-cells of the pars intercerebralis. The significance f this larger amount of material with respect to neurosecretory cell activity is dis-ussed.

INTRODUCTION

NEUROSECRETORY cells in the central nervous system control many major physiological events in the post-embryonic life of insects. The art played by neurosecretory cells in the pars intercerebralis during moulting nd metamorphosis is well known (Wigglesworth, 1954), and in some insects hese cells are also involved in ovarian development and oviposition (E. Thom-en, 1952; Nayar, 1958).

Differences in their histological appearance at different times have often een used as evidence that neurosecretory cells are involved in developmental vents (Dupont-Raabe, 1952, 1956; Arvy & Gabe, 1952, 1953 *a*, *b*, *c*, *d*; Arvy, Bounhiol, & Gabe, 1953; Lhoste, 1952; Junqua, 1956; Fraser, 1957, 1959; Kobayashi, 1957; Highnam, 1958). This method of investigation has much to ommend it, since the effect of slight variations in activity of the cells can be tudied, as opposed to the effect of gross disturbances of the hormonal balance n the body engendered by the experimental removal or implantation of the eurosecretory cells.

In general, two kinds of neurosecretory cell can be differentiated in the ars intercerebralis by chrome-haematoxylin-phloxin staining: those that tain blue-black with the chrome-haematoxylin component, and those that tain red with phloxin (M. Thomsen, 1954 *a*, *b*; Nayar, 1955; de Lerma, 1956; Formigoni, 1956; Kopf, 1957). The two kinds of cell are often called A- and B-cells respectively (Nayar, 1955; Kopf, 1957; Kobayashi, 1957; Johannson,

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1958). Further, with paraldehyde-fuchsin, the A-cells may stain deep purple, and the B-cells red or bluish-green (Johannson, 1958; Fraser, 1959). But Nayar (1955) reports that there is no selective staining of A- and B-cells with paraldehyde-fuchsin in *Iphita limbata*, and de Lerma (1956) states that B-cells may contain purple granules after paraldehyde-fuchsin staining. Dupont-Raabe (1956) has shown that the staining reactions of the different cells in phasmids may depend on the fixative used. It would seem that strict uniformity of technique is necessary for the exact comparison of the cell-types present in different insect species.

The corpora cardiaca have been shown experimentally to play some part in the control of developmental events (E. Thomsen, 1952, 1954; Highnam, 1958). It is thought that this control is effected by the release of material which originates in the neurosecretory cells in the brain, and migrates through the nervi corporis cardiaci to the corpora cardiaca, where it accumulates (Scharrer, 1952; E. Thomsen, 1954). The volume of the corpora cardiaca increases during the course of an instar (Brisson, 1949) and this may be due to the accumulation of neurosecretory material within the glands (Arvy & Gabe, 1952; Highnam, 1958). In *Oncopeltus fasciatus* neurosecretory material accumulates in the wall of the aorta (Johannson, 1958). The release of material from the corpora cardiaca may be correlated with the moulting cycle (Arvy & Gabe, 1953 *d*).

It is possible that developmental events may be controlled either by variations in the rate of production of neurosecretory material by the brain, or by variations in the amount of material released from the corpora cardiaca. These mechanisms need not be self-exclusive, and a combination of the two processes might be responsible for the regulation of post-embryonic development. This problem is being investigated in the desert locust, *Schistocerca gregaria* Forskål. The present paper describes the histology of the neurosecretory system in the mature female locust and certain changes which take place in it during the development of the ovaries.

MATERIAL AND METHODS

Newly emerged females were reared in glass-fronted cages 10 × 10 × 12 ins. 25 to a cage, at a temperature of 30 ±2°C and a relative humidity of 60%. They were fed upon freshly cut grass and ground rat cake. A few mature males were kept in the same cages. Under these conditions, the females matured in 3 to 4 weeks. Owing to the rather large individual variation in maturation rates, it was found impossible to predict maturation times from dates of emergence alone. The description of the histology of the neurosecretory system of the mature female which follows is therefore based upon material taken from adults which had terminal oocytes 5 to 7 mm long. These animals varied in age from 19 to 29 days, and had laid no eggs. The immature adults examined were fixed 4 to 6 days after emergence and all had terminal oocytes 1·0 mm or less in length.

Heads were fixed in Bouin's fluid under reduced pressure to collapse the

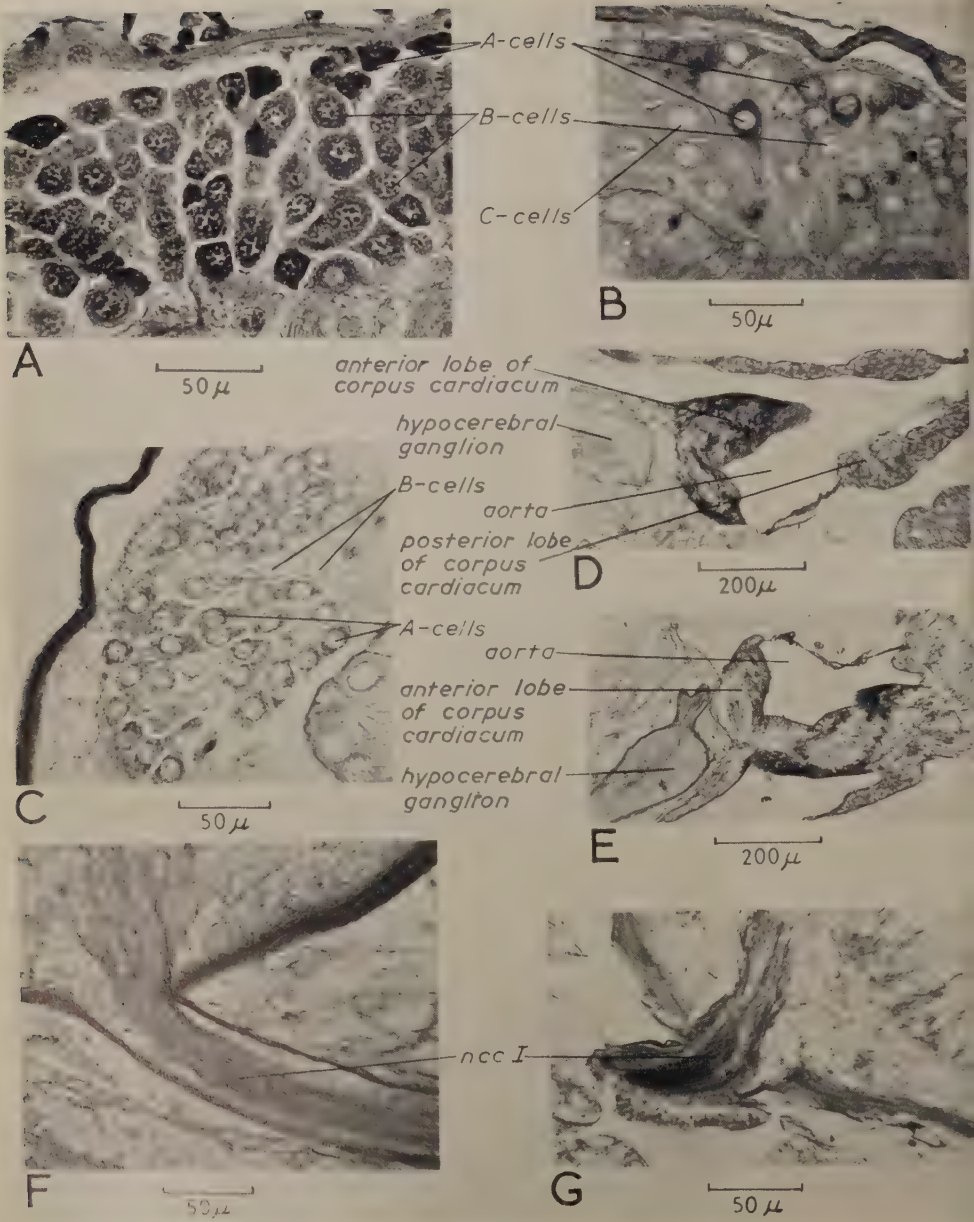


FIG. 1

K. C. HIGHNAM

rge air-sacs in the head and allow rapid and unrestricted fixation of the rain and endocrine system. The cuticle of the head capsule was dissected way before impregnation with paraffin wax. Serial sections were cut at 10 μ nd stained with Gomori's chrome-haematoxylin-phloxin (CHP) or Gomori's araldehyde-fuchsin (PF) after the methods of Halmi (1952) or Dawson 953). The volumes of the neurosecretory cells and corpora cardiaca were lculated from their mean areas, which were measured with the 'Allbrit' Stanley) disk planimeter. Percentages of different cell-types in the neuro-ecretory cell groups were estimated by counting the numbers of cells in very fifth section of a series.

RESULTS

Neurosecretory cells. Two closely apposed groups of neurosecretory cells e present in the dorsal part of the pars intercerebralis region of the proto-erebrum. Each group is about 0·35 mm long by 0·22 mm wide, and extends bout 0·12 mm into the brain near the mid-line. The neurosecretory cells are overed by a layer of vacuolated cells beneath the brain membrane. About ,200 cells may be counted in each group: some 90% of them typically ontain inclusions stainable with CHP and PF.

In the mature female, four types of cell may be identified on the basis of ifferences in size and staining reactions (table 1). About 68% of the cells ain blue-black with CHP. These are the so-called A-cells. Varying amounts f inclusions are found both in the cell-bodies and along their axons (figs. , A, G; 2, A). Where the cells are large, the inclusions are also large and ompletely fill the cell-bodies (figs. 1, A; 2, A). A few of the cells are vacuolated ig. 2, A). When stained with PF, a similar proportion (about 68%) of the ells contain deep purple inclusions (figs. 1, B; 2, A). The inference that these e also A-cells is supported by the examination of alternate sections of the ame series stained with CHP and PF. Many A-cells appear in adjacent ections and the reaction of the same cell to the two stains can be compared.

FIG. 1 (plate). Photomicrographs of sections of various parts of the neurosecretory system f adult female *S. gregaria*. All fixed in Bouin's fluid; A stained with chrome-haematoxylin-hloxin, the rest with paraldehyde-fuchsin.

A, vertical section through the pars intercerebralis region of the brain of a mature female. ote particularly the large A- and B-cells; the A-cells packed with large, deeply staining clusions.

B, as A, but in addition a few C-cells may be seen.

C, vertical section through the pars intercerebralis of the brain of an immature female. ote the absence of large, deeply staining A-cells (compare A and B). The inclusions are nall, and aggregated closely around the nucleus.

D, section through the anterior parts of the corpora cardiaca of a mature female. Note e amount and distribution of the deeply staining neurosecretory material.

E, as D, except that the corpora cardiaca are those of an immature female. The neuro-ecretory material is smaller in amount than in the mature female, and has a more peripheral istribution.

F, section through one of the two nervi corporis cardiaci I at the point where it leaves the rain. Immature female. Note the absence of accumulated material compared with G.

G, as F, but from a mature female. Note the difference in appearance of the nerve compared ith F, caused by the presence of accumulated neurosecretory material.

PF appears to stain the inclusions without colouring the ground cytoplasm c the cell, and consequently the constitution of the inclusions can be mor easily seen than with CHP. It is clear that the large inclusions are actuall aggregates of smaller particles (fig. 2, A). Vacuoles cannot be seen after P staining.

About 17% of the cells stain red with CHP. These are the B-cells. The contain phloxinophil inclusions of small size, never as large as the inclusion within the A-cells. The B-cells vary in size (figs. 1, A, B; 2, B) and some a vacuolated. Their inclusions can be traced along the axons leaving the cell

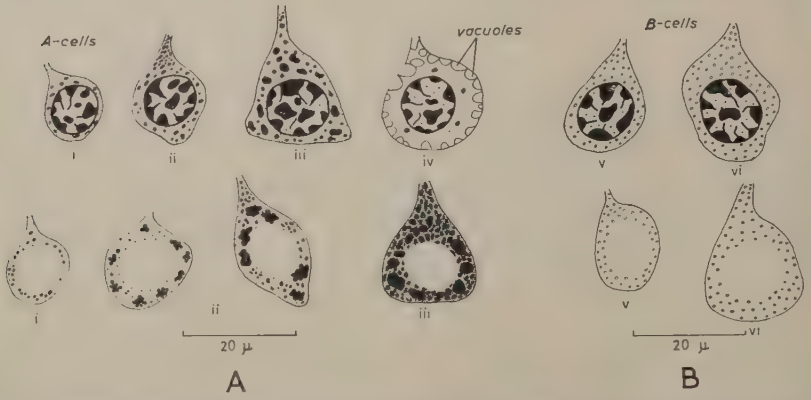


FIG. 2. Camera lucida drawings of A- and B-cells in the pars intercerebralis of adult fem *S. gregaria*. *Upper row*, stained with chrome-haematoxylin-phloxin. *Lower row*, stained wi paraldehyde-fuchsin. A, A-cells, showing differences in size, and differences in the size a the number of inclusions. i and ii are typical of the immature female; iii and iv, in additic are typically found in the mature female. B, B-cells. Typically found in both mature a immature females.

With PF, the B-cells stain a faint pink, or sometimes take on a greenish ting the inclusions are reddish-purple.

Large cells with large nuclei are present near the periphery of each neur secretory cell group (fig. 1, B). They stain a faint purple with CHP and reddi with PF. Inclusions staining blue-black with CHP and red-purple with F are scattered sparsely through the cytoplasm of the cells, but have not be seen along the axons. These cells, called C-cells here, make up about 12 of the total number of cells in each group.

Cells very like the C-cells in their staining reactions, but very much larg (table 1), are found particularly towards the lateral borders of the neur secretory cell groups. These are designated D-cells. Their inclusions, w seen after PF staining, are arranged regularly through the cytoplasm of t cell-bodies, but cannot be seen along the axons. D-cells make up about 5 of the total number of cells in a group. They are very similar in appearan to large motor neurones present in this part of the brain. Neither the C-ce nor the D-cells, in the absence of inclusions along the nerve axons, satis the cytomorphological criteria established by Scharrer & Scharrer (1954 or the identification of neurosecretory cells. They are included in this account nly because their axons run through the brain in company with the axons of he A- and B-cells. A similar association of neurosecretory and non-neuroecretory cells has been found in *Platysamia cecropia* (Stumm-Zollinger, 1957) nd *O. fasciatus* (Johannson, 1958).

In each half of the brain a further group of neurosecretory cells is present etween the pars intercerebralis and the mushroom body, about 0·5 mm rom the mid-line. Each group comprises about 10 cells which have the staining characteristics of A-cells but are larger.

TABLE 1

Characteristics of cells present in the pars intercerebralis of the brain of adult female S. gregaria

Cell type	Nuclear diameter	Cell volume	*Staining reaction*		*Stages present (see fig. 2)*	
			CHP	PF	*immature*	*mature*
A	$10–14\,\mu$	$1·5–5·6 \times 10^3\,\mu^3$	blue-black	deep purple	i, ii	i, ii, iii, iv
B	$10–14\,\mu$	$1·5–6·0 \times 10^3\,\mu^3$	red	pink (sometimes faint green)	v, vi	v, vi
C	$20\,\mu$	$33·5 \pm 2·5 \times 10^3\,\mu^3$	faint purple	pink, reddish-purple inclusions		
D	$23\,\mu$	$47·7 \pm 4·0 \times 10^3\,\mu^3$	faint purple	pink, reddish-purple inclusions		

The nervi corporis cardiaci. The combined axons of the neurosecretory cells n each pars intercerebralis group form a nerve (NCC I) whose path through he brain can be easily traced owing to the presence of stainable material long its length (fig. 1, G). The two nerves pass vertically downwards into the rain for a short distance and then cross one another, all the axons in each erve apparently crossing to the opposite side. Each nerve then continues in a urving path forwards and downwards, running more or less parallel with he anterior face of the pars intercerebralis but at some distance from it, nd finally emerges from the ventral surface of the brain just to one side of he mid-line. Immediately after leaving the ventral surface of the brain, the NCC I bend sharply forwards and pass to the anterior parts of the corpora ardiaca.

The material along the lengths of the NCC I is identical in its staining roperties with the neurosecretory material within the cell-bodies of the pars ntercerebralis. In material stained with CHP, both blue-black and red inlusions can be identified. The blue-black A-material forms aggregates of very much larger size than the similar material within the cell-bodies (fig. 1, G). These large masses are only seen distal to the point of decussation. The red B-material, on the contrary, occurs in small aggregates only a little larger than the inclusions within the B-cells, and only rarely forms large clumps These observations suggest that the two kinds of material differ in physical a well as chemical properties. With PF staining, in sections $10\,\mu$ thick, individua axons may be followed for a considerable distance through the brain, and i can be seen that the A-material may occur at rather regular intervals alon the axon. This may indicate a phasic discharge of the material from the cell body, although it may be a fixation artefact.

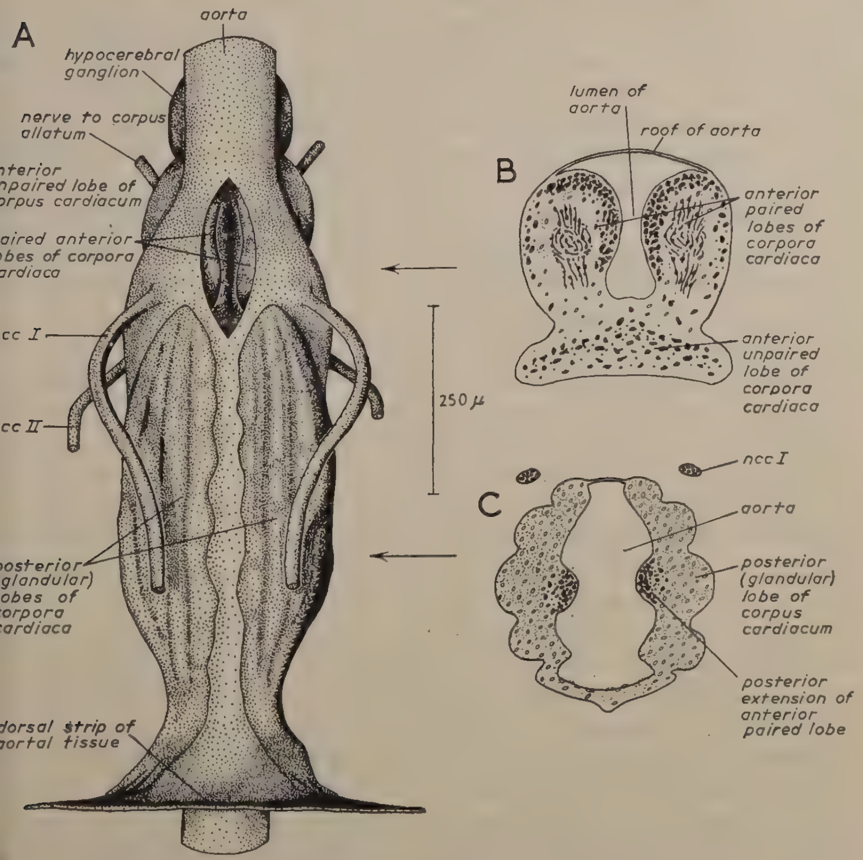
It has not been found possible to trace through the brain the paths of th nervi corporis cardiaci II from their origin in the lateral groups of neuro secretory cells, owing to the absence of stainable material along the axons a this time. This implies that these neurosecretory cells are not dischargin material in the mature female locust. The NCC II can be followed as distinc nerves from the ventral surface of the brain to the anterior parts of the corpor cardiaca.

The corpora cardiaca. These are elongated paired bodies lying behind th brain, dorsal to the hypocerebral ganglion (fig. 3, A). They are intimatel associated with the walls of the aorta, anterior to the point where the vesse bends downwards beneath the brain, following the course of the oesophagus

The anatomy of the corpora cardiaca (oesophageal ganglia) and their rela tion with the aorta and sympathetic nervous system has been described fo *Locusta migratoria* by Albrecht (1953). His description differs in severa details from that given below for *S. gregaria*. Albrecht based his descriptio upon dissected material alone, whereas the following description is amplifie and supported by a detailed examination of histological material. *L. migratori* heads have also been examined histologically, and the structure of the endo crine system has been found to differ very little from that of *S. gregaria*.

With the insect in its normal posture, the long axes of the corpora cardiac run almost vertically, owing to the hypognathous structure of the locus head. The 'posterior' parts of the corpora cardiaca are therefore actuall dorsal to the 'anterior' parts. However, the terms anterior and posterior ar retained in this description in preference to ventral and dorsal, to allow cleare comparison with the glands of other insects.

The posterior ends of the glands are delimited by a transverse strip c tissue originating from the dorsal wall of the aorta (fig. 3, A). This divides int median and lateral strands (Albrecht, 1953), which are inserted into th dorsum of the brain and the anterior horns of the tentorium. A small pouc is formed dorsally to the aorta, where this tissue originates, and the posteric ends of the corpora cardiaca follow the walls of the pouch rather than th main course of the vessel. The corpora cardiaca constitute the lateral wall and the greater part of the ventral wall of the aorta anterior to the region c bending of the vessel. Anteriorly, the corpora cardiaca form two large lobes which project into the lumen of the aorta (fig. 3, A, B), and fuse ventrally t form an unpaired lobe which makes up the whole of the ventral wall of th aorta in this region (fig. 3, B). This ventral unpaired lobe projects a shor distance in front of and behind the dorsal paired lobes; it lies almost imme ately above the hypocerebral ganglion, to which it is connected by a pair of ort nerves. The NCC I and II enter the paired anterior lobes of the corpora rdiaca (fig. 3, A), and the nerves to the corpora allata leave the anterior unired lobe (fig. 3, A). The posterior parts of the corpora cardiaca in the lateral


A
aorta
hypocerebral ganglion
nerve to corpus allatum
nterior npaired lobe of orpus cardiacum
aired anterior bes of corpora ardiaca
cc I
cc II
osterior glandular) obes of orpora ardiaca
dorsal strip of ortal tissue
250 μ
B
lumen of aorta
roof of aorta
anterior paired lobes of corpora cardiaca
anterior unpaired lobe of corpora cardiaca
C
ncc I
aorta
posterior (glandular) lobe of corpus cardiacum
posterior extension of anterior paired lobe

G. 3. A, corpora cardiaca and associated structures of adult female *S. gregaria*. Semigrammatic. Part of the dorsal wall of the aorta is shown cut away to reveal the anterior red lobes of the glands beneath. Explanation in the text. B and C, drawings of sections ough the corpora cardiaca at the levels marked by the arrows. Note the clear differentiation between anterior and posterior parts of the glands.

rta walls do not project into the lumen of the vessel (fig. 3, C); their outer rfaces are folded and lobed (fig. 3, A, C). These posterior parts of the glands most easily seen in fresh dissections, and seem to be the parts figured as mplete corpora cardiaca by Albrecht (1953) in *L. migratoria*.

Histologically, the anterior and posterior parts of the corpora cardiaca are ikingly differentiated by the distribution of stainable material from the urosecretory cells in the brain (figs. 1, D; 3, B, C). With CHP, the anterior ired and unpaired lobes contain large amounts of A-material stained blue-black; this is particularly abundant where the NCC I enter, and also around the periphery of the glands (fig. 3, B). A-material is found also in two finger-like processes of the paired anterior lobes which project backwards along th inner sides of the posterior parts of the glands (fig. 3, C). Other than thes narrow extensions, the posterior parts of the corpora cardiaca contain no A-material. *L. migratoria* has also been examined and exhibits a similar distribution of A-material in the corpora cardiaca of the adult female. This contrast with the report of Nayar (1954) that this species gives a negative reaction wit chrome-haematoxylin. After PF staining, a deep purple colour is distribute in the same way in the corpora cardiaca of both *S. gregaria* and *L. migratoria*

In addition to the A-material, phloxinophil material is present in th anterior lobes of the glands after CHP. It is most obvious in the central part of the anterior lobes, but its presence in the more peripheral regions may b obscured by the deeply staining A-material. The phloxinophil material i thought to be derived from the B-cells in the pars intercerebralis, since it i also present along the lengths of the NCC I.

Phloxinophil material is found in addition in the posterior parts of th corpora cardiaca, but here it is intracellular and is consequently thought t be different in origin from the similarly staining material in the anterior lobe of the glands: it is probably an intrinsic secretion of the corpora cardiaca The rather sharp demarcation between those parts of the corpora cardiaca o *S. gregaria* which store material from the neurosecretory cells in the brai and those parts which produce the intrinsic secretion of the glands shoul make it easier to determine the physiological functions of the two kinds secretion in this species, than in those insects where the glandular cells of th corpora cardiaca are very closely associated with the storage regions, as i *Mimas tiliae* (Highnam, 1958). This possibility is at present being investigate

With CHP, A-material can be traced for a short distance along the nerve to the corpora allata, but never into the glands themselves. With PF, dee purple material can be traced for a similar distance along the nerves, but thi then gives way to a diffusely staining reddish-purple material which can b followed into the corpora allata. This reddish-purple staining may indicat the transformation of the deep purple-staining material into one with differen chemical properties, or it may indicate the presence of material from the cells in the pars intercerebralis, the presence of which in the proximal parts the nerves to the corpora allata is obscured by the deeply staining A-materia Similar observations have been made in other insects (Stutinsky, 1952; Arv Bounhiol, & Gabe, 1953 *b*, *c*; M. Thomsen, 1954 *a*, *b*). Neurosecretory materi can also be traced along the lengths of the short nerves connecting the anteri unpaired lobe of the corpora cardiaca with the hypocerebral ganglion.

The neurosecretory system in the immature female. Changes in the histologic appearance of the neurosecretory cells in the brain during the development the ovaries have previously been described in other insects (Dupont-Raab 1952; Arvy, Bounhiol, & Gabe, 1953; Formigoni, 1956). In *S. gregaria*, cauter of the neurosecretory cells in the pars intercerebralis prevents ovaria levelopment (Highnam, unpublished). The neurosecretory system of imnature females was consequently examined in order to compare it with that of the mature female and determine whether any histological differences ould be related to the neurosecretory control of ovarian development. Making due allowance for the individual variation in maturation times, one an see that the progress of ovarian development is accompanied by a gradual hange in the histological appearance of the system in the immature form to hat described for the mature form.

In the newly emerged female, the neurosecretory cells in the pars intererebralis are not so obvious as in the mature female (fig. 1, C), but this is nly a matter of degree. A-cells may be identified with certainty with both CHP and PF staining. They constitute about the same proportion (68%) of he total number of cells as they do in the mature female. But no large cells packed with large inclusions can be seen at this time. All the cells are relatively mall, with small inclusions arranged mainly around the nucleus (fig. 1, C). This arrangement is best seen with PF staining. The cells are indistinguishable rom the smaller A-cells in the mature female (fig. 2, A). B-cells also occur in he same proportion in the pars intercerebralis of the immature female as in he mature form. Their size, the number and appearance of their inclusions, tc., are very similar to those of the B-cells in the mature female. C- and D-ells are identical in number and appearance with those of the mature female. The only obvious histological differences between the neurosecretory cells of mature and immature females occur in the A-cells of the pars intercerebralis.

The NCC I in the immature female present a further difference in appearnce compared with those in the mature form. The large accumulations of A-material along the lengths of the NCC I, so typical of the mature female (fig. , G), are not found in the immature (fig. 1, F). Close examination of the nerves in the immature female reveals that A-material is present, but always in the form of *small* droplets or particles. This difference is also reflected in the orpora cardiaca: they are smaller in volume than those of the mature female, and the neurosecretory material is less concentrated and aggregated into maller masses (fig. 1, E; compare fig. 1, D). B-material appears to be present in larger amount, both along the NCC I and in the corpora cardiaca of the immature female, but this appearance is probably due to a reduction in the masking effect of the A-material which is present in smaller amount. The implication of this observation is that the B-cells of the pars intercerebralis how little change in activity during ovarian development compared with the A-cells. With PF staining, diffuse reddish-purple material can be traced into the corpora allata in the immature female, as in the mature form.

DISCUSSION

The pars intercerebralis region of the protocerebrum of different insects may contain a large number of small, or a small number of large, neurosecreory cells. There is probably a fairly constant relationship between total eurosecretory cell volume and body-size. This variation in the number and size of neurosecretory cells in different insects makes a strict comparison of the nature and function of various cell-types rather difficult. *S. gregaria* possesses a very large number of small neurosecretory cells, being similar in this respect to various phasmid (Dupont-Raabe, 1952) and hymenopteran (M. Thomsen, 1954 *b*) species.

In *S. gregaria*, A- and B-cells are clearly differentiated with CHP. In addition, the A-cells stain purple with PF, and the B-cells red, sometimes with a greenish tinge. The cells are therefore very similar to the A- and B-cells in the pars intercerebralis of *O. fasciatus* (Johannson, 1958), *Drosophila* (Kopf, 1957), and *Bombyx* (Kobayashi, 1957). Two additional kinds of cell, C- and D-cells, are also present in this part of the brain in *S. gregaria*. The reaction of these cells to PF is rather different from that of the C- and D-cells described by Johannson (1958) in *Oncopeltus*. It is doubtful whether these cells are neurosecretory in *S. gregaria* (p. 30). Johannson (1958) similarly considers that the D-cells of *Oncopeltus* may not be neurosecretory. It is possible that the C- and D-cells are concerned with some mechanism which releases stored neurosecretory material from the corpora cardiaca, since in *S. gregaria* the axons of these cells accompany those of the A- and B-cells to the glands.

The B-cells have been thought to be a stage in the secretory cycle of the A-cells (M. Thomsen, 1954 *b*; de Lerma, 1956; Herlant-Meewis & Pacquet, 1956; Kopf, 1956). Nayar (1955), who examined the reaction of the neurosecretory cells of *I. limbata* to CHP after various experimental treatments, has suggested that the B-cells are actually A-cells deprived of the bulk of their stainable inclusions. This view is supported by the observation of M. Thomsen (1954 *b*) that both red and blue inclusions may be present in the same cell after CHP staining in some Hymenoptera. In *S. gregaria*, both A- and B-cells vary in size, and the different sizes of each cell-type may well represent stages in the elaboration of its own particular inclusion. Further, after CHP staining, red as well as blue-black material can be traced along the axons of the NCC I; and this suggests that both A- and B-cells are discharging their respective inclusions. In mature female *S. gregaria*, where the activity of the A-cells appears to be very different from that in the immature form, no difference in the ratio of A-cells to B- is found. These observations indicate that in *S. gregaria* the B-cells are different in kind from the A-, and are not simply a stage in the secretory cycle of the latter. This conclusion is supported by Johannson (1958), who subjected *O. fasciatus* to a variety of treatments but was unable to detect any transformation from B-cells to A-.

The presence of many large inclusions in the A-cells has usually been thought to indicate that the cells are more active than when the inclusions are few and small (Dupont-Raabe, 1952; Arvy & Gabe, 1952; Formigoni, 1956; Herlant-Meewis & Pacquet, 1956). On this interpretation the difference in appearance of the A-cells in *S. gregaria* in immature and mature females would seem to indicate that the cells are secreting more actively in the mature insects, and that more material is transported along the NCC I to the corpora cardiaca. This interpretation, however, needs to be carefully examined.

The amount of material contained in a neurosecretory cell at any time depends both upon its rate of synthesis and its rate of discharge. The observaion that at times some neurosecretory cells contain larger amounts of material han at others may therefore indicate not an increased rate of synthesis, but a decreased rate of discharge, with a consequent accumulation of material within the cell-bodies. In mature *S. gregaria*, this accumulation of material within the A-cells may be a reflection of accumulation in the corpora cardiaca and along the NCC I. In effect, the amount of material in the cell-body is no ndication of its *rate* of secretion. Further, material from the neurosecretory cells is discharged along the NCC I to the corpora cardiaca in the immature emale locust, as well as in the mature female, so that the large cells packed with inclusions found in the mature female are not necessarily an essential stage in the secretory cycle of the A-cells.

Another factor that may still further complicate the picture is the duration of the secretory cycle. If this changes, by being speeded up or retarded, the output of the neurosecretory cells (in a given time) could increase or decrease without in any way affecting the histological picture. It is significant that no *histological* differences exist between the neurosecretory cells of *Rhodnius prolixus* before and at the height of secretion of the brain hormone, the imes of which had been precisely determined experimentally (Wigglesworth, 1940).

It is concluded, therefore, that the histological differences which exist between the neurosecretory cells in the pars intercerebralis of immature and mature *S. gregaria* do not provide sufficient evidence for either increased or decreased activity at these different stages in development. However, the observations presented here do suggest that neurosecretory material accumulates in the corpora cardiaca of the mature female, and there is a strong possibility that the material accumulates also along the NCC I and within he A-cell-bodies. Further information concerning the importance of this material for oviposition and the subsequent development of more oocytes will be presented in a later paper.

It should be remembered that the stainable material within the neurosecretory cells and the corpora cardiaca is not necessarily identifiable with any hormonally active principle (Scharrer & Scharrer, 1954 *a*). The stainable material is thought to be a 'carrier protein' for the actual brain hormone (or hormones). The assumption is made that when the stainable material is present, so also is the hormone.

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The Histochemistry of the Male Germ-cells of the Nematode *Porrocaecum angusticolle*, a Parasite in the Vulture

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With two plates (figs. 1 and 2)

SUMMARY

The cytoplasm of the male germ-cells of *Porrocaecum angusticolle* contains (1) iospholipid granules, (2) 'refringent bodies' consisting of ribonucleoproteins at first, it some masked lipids as well in the late spermatid, (3) mitochondria of the usual ooprotein nature. The refringent bodies arise in the spermatocytes in close associa- on with the phospholipid granules ('Golgi bodies') by the aggregation of cytoplasmic isiphil material. In the spermatids the refringent bodies gradually fuse to form a ngle cone-like structure which occupies the narrow posterior region of the sperma- zoon.

This cone-like structure, judging from its origin in close association with the 'Golgi odies' (acroblasts), is the homologue of the acrosome of a normal flagellate sperm; it it is completely devoid of polysaccharides and seems unable to function as an rosome, since it occupies a position posterior to the nucleus in the spermatozoon. n the contrary, some polysaccharides have been demonstrated by the authors in the road anterior region of the spermatozoon, which may be functioning as an acrosome.

INTRODUCTION

NEMATODE spermatogenesis has been studied by a number of cytologists. Some of the previous work on the subject has been reviewed by owen (1925), Sturdivant (1934), and Nath and Singh (1956).

The nematode spermatozoon is characterized by the presence of a remark- ble structure—a large, conical, refractile body, variously called the 'refringent ody', the 'ascaridine granule', the 'acrosome', &c. The homology of this ther unique structure with the acrosome of a typical flagellate sperm was rst suggested by Bowen (1925). This author considered the refringent body the nematode sperm as a differentiated product of the 'Golgi material', ke the acrosome of the typical spermatozoon. This homology has been con- rmed by some of the later workers (Sturdivant, 1934; Collier, 1936; Nath Singh, 1956; Singh, 1957; Hovasse, 1958).

It has been found during the past few years that the acrosome of the agellate sperm of widely different groups of animals is characterized by the resence of a mucopolysaccharide, demonstrable by the periodic acid / Schiff action (Wislocki, 1949; Leblond, 1950; Schrader & Leuchtenberger, 1951; lermont & Leblond, 1955; Moriber, 1956; Clayton & others, 1958; &c.).

There have been various speculations about the chemical composition of e refringent bodies of the nematode spermatozoon. Since these bodies

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appear highly refractile in the living condition, they were usually supposed to consist of a fatty substance.

Romieu (1911), however, considered the refringent bodies as consisting of 'nucleo-albumen'. This observation has recently found support in the histochemical studies of Pasteels (1948), Panijel and Pasteels (1951), Delavault (1952), and Favard (1958), who stated that these bodies consisted of ribonucleoproteins. Similarly, Makarov (1958) has denied the presence of any polysaccharides in these structures.

It may, therefore, be concluded from this brief review that chemically the refringent body of the nematode spermatozoon bears no resemblance to the acrosome of a flagellate spermatozoon.

The origin of the refringent body has been also disputed (see Sturdivant, 1934; Nath & Singh, 1956; Hovasse, 1958). Very recently Favard (1958) has put forward evidence from electron microscopy that the ascaridine granules (refringent bodies) of the spermatocytes of *Parascaris equorum* arise from the 'grains de Palade' of the ergastoplasm. The lipid inclusions represent simply the sites of this synthesis; they disappear during the formation of the ascaridine granules.

It was considered worth while to undertake a histochemical study of spermatogenesis in a nematode to ascertain the chemical nature, origin, and probable homology of the refringent body.

MATERIAL AND TECHNIQUE

Porrocaecum angusticolle is a parasite in the intestine of the vulture. Th parasites were removed from freshly killed vultures and dissected in paraffin lined dishes containing physiological saline (Baker, 1944). The testis, which is a single, coiled, thin thread about 5 to 8 cm in length, was transferred immediately to various fixatives. The uteri of the females were fixed fo study of the ripe spermatozoon.

The various histochemical techniques employed have been listed in th appendix to this paper. Some of the paraffin sections of material fixed i various ways were stained with Heidenhain's iron-haematoxylin. The aci fuchsine technique of Cain (1948), the 'Hermann PO' technique of Bake (1957), and the Kolatchev technique were also employed.

Living cells were studied by phase-contrast microscopy, and with supervita dyes like neutral red and Janus green B.

RESULTS

The cytoplasm of the male germ-cells of *P. angusticolle* shows the followin inclusions:

1. Lipid bodies (granules and duplex spheres), consisting of phospholipids
2. 'Refringent bodies', rich in ribonucleoproteins and developing som masked lipids also, in later stages.
3. Mitochondria, consisting of lipoproteins.

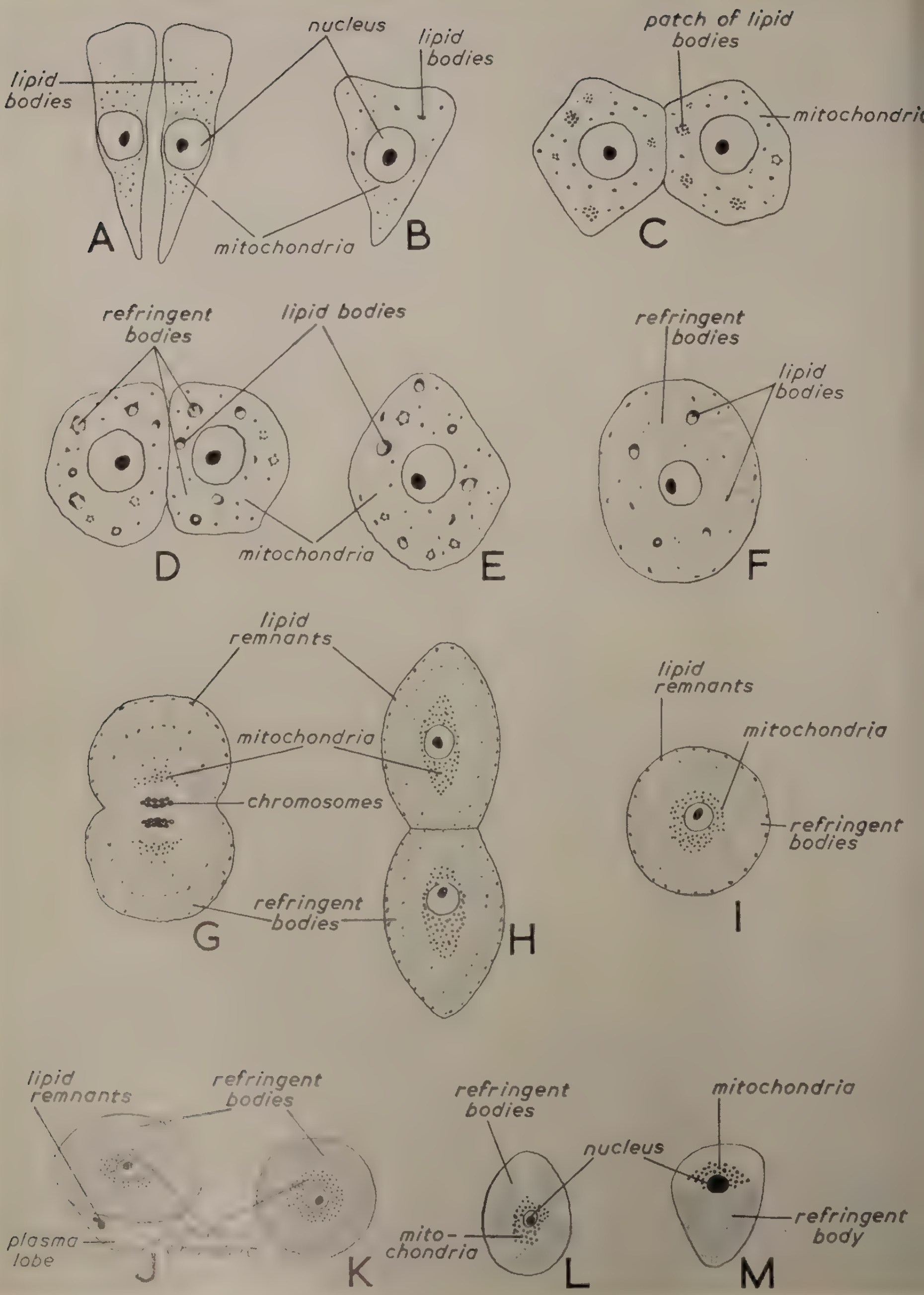

FIG. 1

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The morphology, histochemistry, and ultimate fate of these inclusions in ɔermatogenesis has been studied. The results of the various histochemical ests employed have been given in the appendix to this paper; only the elevant conclusions drawn from such tests are given here. Figs. 1 and 2 resent process of spermatogenesis diagrammatically.

permatocytes

The earliest spermatocyte appears as an elongated, conical cell with a entrally placed spherical nucleus containing a single prominent nucleolus ig. 1, A). The cytoplasm and the nucleolus are intensely basiphil. The various xtraction-controls show that the cytoplasmic basiphilia is attributable to NA. Besides this, some minute mitochondria can be seen, forming an ggregation round the nucleus, both in the fixed and living cells. A few minute hospholipid granules are dispersed in the cytoplasm. However, the marginal ytoplasm is free from any such inclusions.

The spermatocyte now enters the growth phase and gradually assumes a olyhedral shape in sections (fig. 1, B, C). The mitochondria, which now appear s distinct fibrils, become uniformly dispersed (C). They can easily be demonrated in living cells under the phase-contrast microscope, or with Janus reen B, and in the processed material by various techniques (acid haematein AH), iron-haematoxylin applied to Lewitsky-fixed gelatin sections, acid ıchsine (Cain, 1948), &c.). They are phospholipid-protein in nature. They o not show any distinct basiphilia. The nucleus remains centrally placed and rows slightly. It is very uncommon to find the stages of regular prophase; ıost of the cells encountered are in the resting or growth phase (C).

The phospholipid granules proliferate and grow slightly. Occasionally some f these granules appear as short straight or curved rods (B); this is probably ue to the fusion of individual granules. Very soon these granules arrange ıemselves in small groups; and 4 or 5 such groups can be seen in each permatocyte (C). However, separate phospholipid granules are also abundant. ;ytoplasmic basiphilia continues to be intense.

The formation of refringent bodies is ushered in by the appearance of small pherical spaces in the centre of the phospholipid aggregations (C); the lipid ranules become applied to the surface of these vacuole-like spaces, thus giving he appearance of the typical 'mulberry spheroids' of Thomas (1948). A trongly basiphil material quickly fills this vacuole-like internum of the ıulberry spheroids. This material represents the refringent body or ascaridine

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FIG. 1 (plate). A–M, diagrammatic representations of the process of spermatogenesis in *'orrocaecum angusticolle*. The diagrams are based on the study of the living as well as processed material.

A, earliest primary spermatocyte.
B–F, subsequent stages in the development of the primary spermatocyte.
G, first maturation division.
H, second maturation division.
I–L, stages of spermateleosis.
M, ripe spermatozoon.

granule (D). The refringent body grows into a separate sphere about 1 μ i diameter; it gets rid of its phospholipid 'satellite' granules, and comes to l free in the cytoplasm. Very commonly the phospholipid material appears i the form of an irregular thickening or a crescent, or even a ring around th developing refringent body (D, E). These appearances are due to the confluenc of the individual lipid granules. The formation of new refringent bodies continues till the whole of cytoplasm of the spermatocyte becomes filled up wit them (F). The lipid remnants in the form of small granules or short straight c curved rods lie interspersed among these refringent bodies.

It is very difficult ordinarily to locate any mitochondria in this stage wit any of the techniques, owing to an overcrowding of the refringent bodies i the cytoplasm (F).

The single nucleolus and the nucleoplasm do not show any basiphil while the refringent bodies are being formed (fig. 2, A). The nucleolus eosinophil.

While the formation of the refringent bodies proceeds in the cytoplasm, th latter gradually loses its basiphilia. During prometaphase I and the subsequen stages the cytoplasm of the cell is almost completely negative to the variou basic dyes.

During the prometaphase and the subsequent stages of meiosis I and I which appear in quick succession without any resting stage, the refringen bodies appear slightly elongated and oval; they are arranged in the form c three or four concentric rings in the cytoplasm (fig. 1, G).

The mitochondria now assume a spherical appearance; they occupy th two poles of the spindle during meiosis (G). They become more sudanoph and more AH-positive, but remain unchanged in their general histochemic nature.

Spermatids

The spermatid receives more or less equal quantities of all the three cytoplasmic inclusions. The refringent bodies continue to be arranged in thre concentric rings. Some clear cytoplasm is left around the nucleus; this occupied by the granular mitochondria. All the remaining phospholipi granules, on the contrary, move to the extreme periphery of the cell (fig. H, I). The nucleus is small and spherical and continues to show a singl prominent nucleolus. Now there occurs the well-known process of plasma lobe-formation, in which the excess of cytoplasm along with all the lipi remnants is sloughed off. The cytoplasm of the spermatid and of the plasma lobe gives a distinct PAS-positive reaction, owing to the presence of polysaccharide.

Before moving into plasma-lobe, the phospholipid granules fuse to form a irregular mass (J). Simultaneously, the refringent bodies begin to fuse wit each other to form a tight ring of slightly elongated, cuboidal structures i the maturing spermatid (K). With a further process of condensation of the ribonucleoproteins of the refringent bodies, 5 to 6 large spheres are formed (L

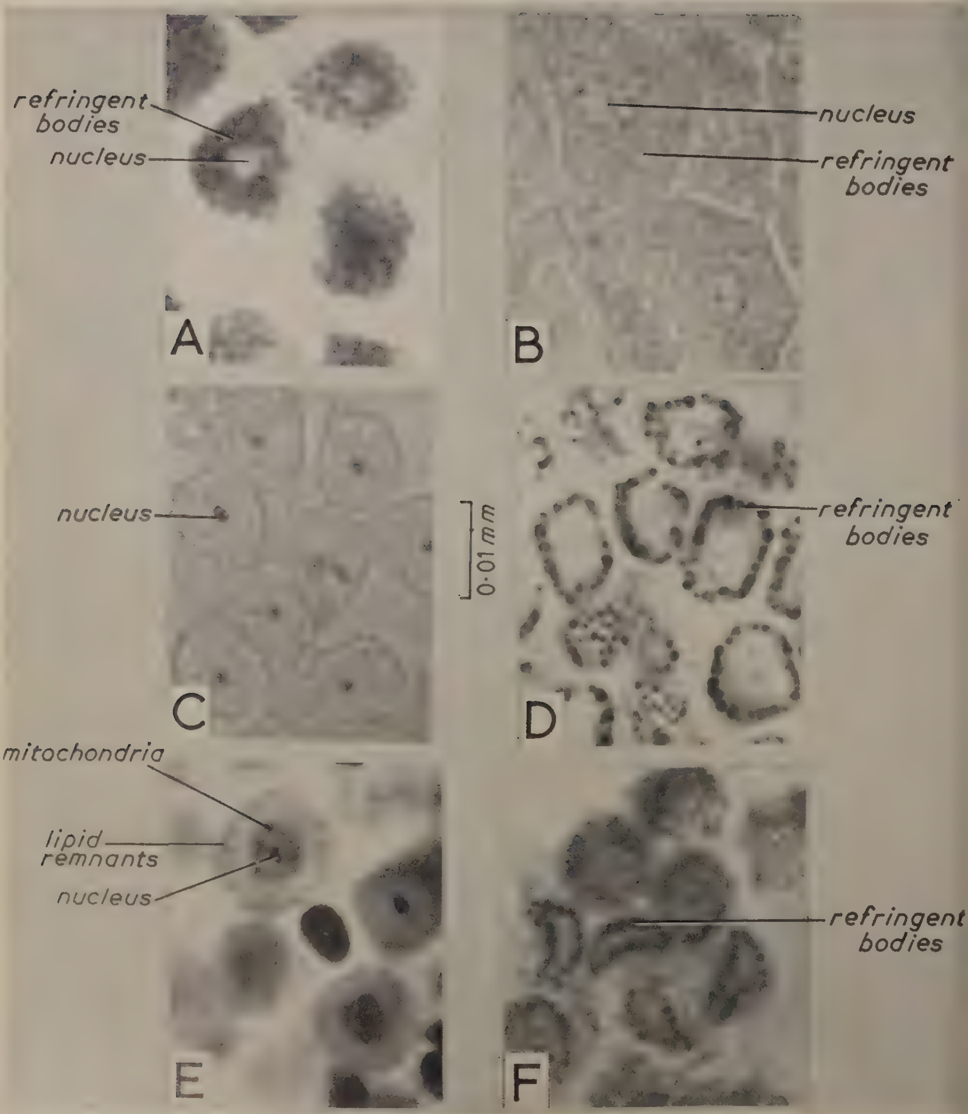

FIG. 2

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imately all of them fuse to form a single conical refringent body in the
e spermatozoon (M). The mitochondria continue to be situated round the
cleus (fig. 2, E). They become larger and fewer, apparently by fusion of
smaller granules. The nuclear material also becomes condensed into a
all, spherical, Feulgen-positive mass (figs. 1, M; 2, C).

The circumnuclear region of the cytoplasm of the maturing spermatids
l the spermatozoon gives a slight PAS-positive reaction without any meta-
omasy. However, it is difficult to say whether the polysaccharide is present
the mitochondria of this region or in the ground cytoplasm, or in both.

The histochemical changes in the refringent bodies during the process of
turation are interesting. It has already been pointed out that these bodies
strongly basiphil, owing to the presence of large amounts of ribonucleo-
teins (fig. 2, A, B). However, as soon as the refringent bodies arrange them-
ves in a single ring in the maturing spermatid, they suddenly lose all their
iphilia in routine preparations. These stages are by far the most abundant
the testicular material of *Porrocaecum*. However, in the ripe spermatozoon
ich is found only in the uterus of the female), the single refringent body
in develops slight basiphilia.

t has been mentioned earlier that there are no histochemically demon-
ble lipids in the refringent bodies till the early spermatid stage. The
ious unmasking techniques given in the appendix also do not impart any
anophil coloration to these structures. However, the burnt Sudan black B
nique of Berenbaum (1958) colours quite intensely the refringent bodies
the maturing spermatid, particularly at the stage when they lose their
iphilia (fig. 2, F). The chromosomes of all the stages are also strongly
ured. This would show that the refringent bodies during the later stages
maturation develop some quantities of lipids, probably in the form of a
protein complex.

t has been further observed that the sudden loss of basiphilia by the
ingent bodies at a particular stage of the spermatid is not due to the
ence of ribonucleoproteins as such, but rather to their masking by a lipo-
tein complex, as shown by the following experiment. When paraffin
ions of material fixed in Zenker (3 h) or in Carnoy are treated with 4%
hloroacetic acid (TCA) at 90° C for 15 min. as a control for RNA, the
ingent bodies of all other stages except the maturing spermatids lose their

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IG. 2 (plate). Photomicrographs of the male germ-cells of *Porrocaecum angusticolle* from
ous histochemical preparations.

spermatocyte from a methyl green / pyronin G preparation, showing pyroninophil
ngent bodies.

same as in A but after digestion in 4% TCA for 15 min.

spermatids after Feulgen nucleal reaction.

spermatids from methyl green / pyronin G after extraction in 4% trichloroacetic acid
h.

spermatids from Lewitsky-saline gelatin sections stained with 0·5% iron haematoxylin.

spermatids from Berenbaum's burnt Sudan black B preparation, showing sudanophil
ngent bodies.

basiphilia (fig. 2, B). The refringent bodies of the maturing spermatids on the contrary, develop slight basiphilia after this treatment, although they are not basiphil in ordinary preparations. When the slides are kept in TCA for longer periods, the basiphilia of these refringent bodies increases progressively; they become strongly basiphil after a treatment for 1 h (fig. 2 D). A further increase in the time of digestion in TCA causes a rapid loss o basiphilia from these bodies, till they become completely non-basiphil.

It is, therefore, logical to conclude that the loss of basiphilia of the refringent bodies during the maturation stages of the spermatid is due to some kind of masking of RNA, perhaps by the formation of a complex containing proteins, lipids, and RNA.

It is now generally accepted that digestion by TCA breaks the RNA/lipoprotein bond and thus causes the extraction of RNA from the cell. But in this particular material TCA first causes an unmasking effect on the refringen bodies, perhaps by breaking the complex mentioned above, and later causes the extraction of RNA.

A similar effect of digestion by TCA has also been noticed in the refringen body of the ripe spermatozoon found in the uterus.

The ripe spermatozoon of *Porrocaecum* is of typical ascarid type, being cone-like. The nucleus along with the mitochondria occupies the anterio blunt end, while the conical refringent body fits into the posterior pointed hal of the spermatozoon.

It has been further confirmed that during fertilization the spermatozoon enters the egg with its blunt end forward. When the whole spermatozoon ha entered the ooplasm, the refringent body becomes rounded. Gradually it material disappears in the cytoplasm of the egg.

DISCUSSION

The ripe spermatozoon of *P. angusticolle* is of typical ascarid type (Nath and Singh, 1956). The most prominent structure in this spermatozoon is the large 'refringent body', also called the 'acrosome' (Bowen, 1925; Sturdivant 1934; Collier, 1936; Nath & Singh, 1956; Singh, 1957; Hovasse, 1958) o the 'ascaridine granule' or 'body' (Pasteels, 1948; Panijel & Pasteels, 1951 Favard, 1958). This refringent body occupies about two-thirds of the cone-like spermatozoon, lying behind the nucleus towards the narrow posterior end of the cone.

The chemical nature of the refringent body has been variously described in the earlier work (Bowen, 1925; Sturdivant, 1934; &c.). Quite recently Pasteels (1948), Panijel and Pasteels (1951), Delavault (1952), Favard (1958), and Makarov (1958) have produced evidence to show that it contains ribonucleoproteins.

Our observations on *Porrocaecum* spermatozoon fully confirm the presence of ribonucleoproteins in the refringent body. In addition, we have also found some small quantity of masked lipids in the refringent body o the maturing spermatid. The lipids seem to be present more or less in the isked state described recently by many workers (Berenbaum, 1958; Clayton, 58; Chayen & others, 1959).

Pasteels (1948) observed that the refringent bodies (his 'ascaridine granules') e their basiphilia suddenly in the maturing spermatid and do not regain it fertilization. Our observations also reveal a similar behaviour of the ringent bodies in normal preparations. However, treatment of the sections 4% TCA at 90° C for 1 h completely restores the capacity of these bodies stain with pyronine G. A further prolongation of extraction, even for a few nutes, again removes the basiphilia. This indicates that the RNA in these dies is present at this stage in some complex form with lipoproteins; i.e. it 'masked' and not absent, as Pasteels believed (1948).

The single conical refringent body of the ripe spermatazoon is formed by gradual fusion and condensation of the material of the smaller spherical dies which arise in the primary spermatocyte. In fact, the major cytoplasmic ivity of the male germ-cells in this species seems to consist of the formation these structures. Besides this the spermatid shows very little activity while nsforming into the mature spermatozoon, except that small quantities of the essive cytoplasm along with lipid remnants are cast off as the plasma-lobe.

Our observations fully support the conclusion of previous authors that the tochondria of the spermatocyte, which are in the form of filaments and nules, do not play any direct role in the formation of the refringent bodies urdivant, 1934; Collier, 1936; Nath & Singh, 1956; Hovasse, 1958; vard, 1958).

Our observations lead us to believe that the refringent bodies arise by consation of the cytoplasmic basiphil substances (ribonucleoproteins) into heres. In the early spermatocyte the cytoplasm, containing sparsely disbuted mitochondria and phospholipid granules, is intensely basiphil, the siphilia being attributable to the presence of RNA (which is labile to ivary ribonuclease).

As the process of spermatogenesis advances and the spermatocyte enters the wth period, small vacuoles or spaces appear within the localized aggregans of phospholipid granules; the latter become attached to the surface of former. These vacuoles or spaces become quickly filled up with the basiphil terial (ribonucleoproteins). Gradually the ribonucleoprotein sphere grows; imately it gets rid of its phospholipid satellites and forms a refringent body. hen the primary spermatocyte approaches the prometaphase stage, its oplasm has become choked with these refringent bodies, obliterating the tochondria from view. The phospholipids show an obvious decrease during se stages. However, it may be that the phospholipid granules do not ectly contribute any substance in the formation of the refringent bodies; haps they help in the segregation of the dispersed basiphil material by ming lipid membranes. The role of lipids in the formation of such segreion membranes around the secretion products in the various animal cells been stressed by many recent workers, particularly Hirsch (1955).

A point of great interest is that the nucleus of the spermatocyte throughout the process of refringent body-formation contains a single inactive (though prominent) nucleolus. The rest of the nucleoplasm is almost devoid of any pyroninophil substance. The origin of the cytoplasmic RNA is usually attributed to the nucleus (Brachet, 1957; Chantrenne, 1958; Zalokar, 1959). The lack of activity of the nucleolus particularly and of the nucleus as a whole might be interpreted as meaning that no new RNA is synthesized in the nucleus for the formation of the refringent bodies.

Favard (1958), working with the electron microscope, believes that the ascaridine granules in the spermatocytes of *Parascaris equorum* are formed by the aggregation of the 'grains de Palade' of the ergastoplasm or of the endoplasmic reticulum. Such aggregations to begin with are associated with lipid droplets, but become free from them ultimately, forming ascaridine granules Favard (1958) considers these lipids as neutral glyceride droplets.

Bowen (1925), after studying the work of Hirschler (1913) and other investigators of the nematode spermatozoon, was the first to suggest tentatively the homology of the refringent body with the acrosome of other animals. He basec this idea on the fact that the refringent body arises by the confluence of simila spheres which appear in the spermatocyte in association with the 'Golgi bodies' like the acrosome of the flagellate sperm. It must be noted, however, tha Bowen fully realized the shortcomings of such a homology and stressed tha the term 'acrosome' was inadequate to explain the chemical properties anc the physiological role of the structure thus named in the various spermatozoa

Many workers on the spermatozoon of *Ascaris* have accepted this supposec homology (Sturdivant, 1934; Collier, 1936; Nath & Singh, 1956; Hovasse 1958).

In our view Bowen (1925) and almost all other earlier workers correctl considered the 'Golgi bodies' of nematode spermatocytes and the 'Golg dictyosomes' of male germ-cells of other animals as homologous.

The separate phospholipid bodies of the spermatocytes described by us i *Porrocaecum angusticolle* are no doubt the structures that have been describec under the name of 'Golgi bodies' or 'Golgi dictyosomes' by the earlier worker on nematodes (Tretjakoff, 1905; Scheben, 1905; Hirschler, 1913; Sturdivant 1934; Collier, 1936; Nath & Singh, 1956; Hovasse, 1958). Recent work wit the help of electron microscope has shown that the 'dictyosomes' of the mal germ-cells of a large variety of animals have a very characteristic structure o double lamellae associated with some small vesicles, corresponding respectivel to the chromophil and chromophobe regions seen with the light microscop (Burgos & Fawcett, 1955; Clermont, 1956; Palade, 1955; Clayton & others 1958; Gatenby & others, 1958). Favard (1958), however, has found tha the lipid granules of the spermatocytes of *Ascaris* do not have any such characteristic ultrastructure.

A good deal of work has been done recently on the chemical nature of th acrosome of a variety of flagellate spermatozoa, and it has been found tha the acrosome invariably contains some PAS-positive substance, perhap neutral mucopolysaccharides (Wislocki, 1949; Leblond, 1950; Leuchten rger & Schrader, 1950; Schrader & Leuchtenberger, 1951; Clermont & eblond, 1955; Moriber, 1956; Clayton & others, 1958). The acrosome does t contain even the slightest trace of any basiphil substance at any stage lermont, 1956). In fact, the ripe flagellate spermatozoon does not have any preciable quantity of basiphil substances in the cytoplasm. Most of the toplasmic basiphilia or ergastoplasm is discarded during the process of aturation and by sloughing off of the cytoplasm (Daoust & Clermont, 1955).

The refringent body of the *Porrocaecum* spermatozoon, on the contrary, es not contain any PAS-positive substances whatsoever. On the contrary, is solely composed of the cytoplasmic ribonucleoproteins—a substance ich is completely discarded in the normal flagellate sperm.

Again, apart from the fact that the refringent body lies behind the nucleus the ascarid spermatozoon, it gives no indication during fertilization that it itiates the egg-reaction like the acrosome of a typical flagellate sperm. It is nerally believed that the acrosome contains some 'active substances' which lp in the dissolution of the membranes around the unfertilized eggs. These bstances are believed to be present in the form of mucopolysaccharides and oteins (Rothschild, 1958). Even when the acrosome of an atypical flagellate erm like that of *Lepisma* is present behind the nucleus (Bowen, 1925; Nath Bhatia, 1953), it continues to have at least some PAS-positive substances npublished observations).

The cytoplasm and the mitochondrial granules at the anterior blunt end of e sperm of *Porrocaecum* are slightly PAS-positive. Perhaps they contain e enzymes normally carried by the acrosome of the flagellate sperm.

It is quite clear that the refringent body of the ascarid sperm is, at least emically and physiologically, different from the acrosome of the flagellate erm.

It is worth while to mention that some of the very early workers on the matode spermatozoon anticipated the ribonucleoprotein nature of the fringent body. Scheben (1905) considered this body as composed of the hromatic' material of the nucleus. Romieu (1911) considered this structure the 'nucleo-albumen'.

We do not know the function of the refringent body in fertilization. The nior author (Nath) believes that, in view of its origin in close association th the so-called Golgi bodies, the refringent body of the ascarid sperm is mologous with the acrosome of the typical flagellate spermatozoon, but it s undergone clearly a change in function. This has involved changes in its emical composition, morphology, and position in the ripe sperm.

We have already stated that there is overwhelming evidence in favour of the ew that the acrosome of the normal flagellate sperm is the seat of PAS-positive bstances (Rothschild, 1958) which initiate the fertilization reaction on the rt of the egg. If this is true, the broad anterior end of the cone-like sperm of *rrocaecum* in which we have demonstrated some PAS-positive substances ay be functioning as the acrosome. This view is strongly supported by the mplete absence of PAS-positive substances from the refringent body.

APPENDIX

Table showing the histochemistry of the cytoplasmic inclusions during the spermatogenesis of Porrocaecum angusticolle

Technique	Fixation	Embedding medium	Reference	*Spermatocyte*			*Spermatid*		
				Lipid bodies	*Refringent bodies*	*Mito-chondria*	*Lipid bodies*	*Refringent bodies*	*Mito-chondria*
SBB in 70% ethanol; at 60° C; in acetone, hot; after phenol; after 90% ethanol, and dioxane	FCa, FCa+Pc, L, Z, C	P and G	Baker, 1949; Pearse, 1954; Berenbaum, 1958; Clayton, 1958	++	O	+	++	O	++
Burnt SBB	Z, C	P	Berenbaum, 1958	+++	O	+	+++	++	++
AH	FCa+Pc	P and G	Baker, 1946	++	O	+	++	O	++
AH after PE	WB+PE	P	Baker, 1946	O	O	O	O	O	O
NB	FCa, FCa+Pc	G	Cain, 1948	Bl	O	O	Bl	O	O
MG/PG	Z 3 h	P	Jordan & Baker, 1955	O	++	O	O	O	O
MG/PG after saliva	Z 3 h	P	Bradbury, 1956	O	O	O	O	O	O
MG/PG after 4% TCA 15 min.	Z 3 h	P	Pearse, 1954	O	O	O	O	+	O
MG/PG after 4% TCA 1 h	Z 3 h	P	..	O	O	O	O	++	O
MG/PG after 4% TCA 70 min.	Z 3 h	P	..	O	O	O	O	O	O
Eosin/MB	Z 3 h	P	Gatenby & Beams, 1950	O	++Bl	O	O	++Bl	O
Eosin/MB after saliva	Z 3 h	P	Bradbury, 1956	O	O	O	O	O	O
Eosin/GW	Z, B	P	Pearse, 1954	O	++Bl	O	O	++Bl	O
Feulgen	C, B, H	P	Feulgen & Rossenbeck, 1924	O	O	O	O	O	O
Hg-BPB	C, B, Z, FCa	P and G	Mazia & others, 1953	O	O	+	O	O	+
Hg-BPB after PE	WB+PE	G	..	O	O	+	O	O	+

Millon's reaction	Z, C	P	Bensley & Gersh, 1933; Pearse, 1954	O	O (?)	+	O	O (?)	+
CTZ	Z, C, B	P	Danielli, 1947; Pearse, 1954	O	++	+	O	++	+
CTZ after DNFB	,,	P	,,	O	O	O	O	O	O
CTZ after PFA	,,	P	,,	O	+	O	O	+	O
CTZ after benzoylation	,,	P	,,	O	O	O	O	O	O
Sakaguchi	B, C, FCa	P	Baker, 1947	O	O	O	O	O	O
Mercuric sulphate test for phenols	FS	P and celloidin	Baker, 1956	O	O (?)	O	O	O (?)	O
PAS	FCa, B, C	P and G	Hotchkiss, 1948; Pearse, 1954	O	O	O	O	O	+ (?)
Best's carmine	Z, C, B	P	Pearse, 1954	O	O	O	O	O	O
HPO	Hermann +OsO_4	P	Baker, 1957	++	O	+	++	O	+

Key: AH = acid haematein; B = Bouin; Bl = blue; C = Carnoy; CTZ = coupled tetrazonium; DNFB = dinitrofluorobenzene; Eosin/MB = eosin/methylene blue; Eosin/GW = eosin/Gram/Weigert; FCa = formaldehyde-calcium; FS = formalde-hyde saline; G = gelatin; H = Helly; Hg-BPB = mercuric bromophenol blue; HPO = Hermann + post-osmication; L = Lewitsky-saline; MG/PG = methyl green/pyronin G; NB = Nile blue; P = paraffin; PAS = periodic acid/Schiff; PE = pyridine extraction; PFA = performic acid; PC = postchroming; SBB = Sudan black B; TCA = trichloroacetic acid; WB = weak Bouin; Z = Zenker. O = negative; +, ++, +++ = positive; (?) = doubtful.

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Morphological and Histochemical Studies of the Chromatoid Body in the Grass-snake, *Natrix natrix*

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With two plates (figs. 1 and 2)

SUMMARY

The chromatoid body in the spermatogenesis of the grass-snake, *Natrix natrix*, s been studied by the use of phase-contrast microscopy, vital dyes, and histochemical ts.

It first appears during the growth of the primary spermatocyte and is also seen in the condary spermatocyte and late spermatid, but is absent at metaphases of both the turation divisions, in the early spermatid, and during the final stages of ermateleosis. It does not make any visible contribution to the final make-up of the ermatozoon.

In living cells it gives a very low phase change, and is not stained by neutral red or us green. The histochemical study reveals that it consists mainly of RNA and of oteins with abundant acidic and basic groups.

It is tentatively suggested that its function is to provide basic proteins for the final turation of the chromatin in the nucleus of late spermatid.

INTRODUCTION

A CHROMATOID body was for the first time described in a reptile by myself in a study of the spermatogenesis of an Indian snake, *Natrix piscator* ud, 1955). Painter (1921) had previously figured it in the normal and giant ermatids of a lizard, but he neither labelled it nor mentioned it in the text. ox (1952) depicted it in the late spermatids of the garter snake, *Thamnophis* ., but wrongly named it 'acroblast'. I recorded its presence in *Natrix* in the imary spermatocyte and also in the late spermatid, just after the condensaon of the nucleus. In the primary spermatocyte it is rounded or ovoid; in e spermatid at the time of its reappearance it is dumbell-shaped and later herical. At both stages of spermatogenesis it is a large, homogeneous body. the primary spermatocyte an additional smaller chromatoid body and in e late spermatid two or three very small chromatoid bodies are sometimes esent. In the late stages of spermateleosis the chromatoid body moves forrd towards the anterior end and disappears.

In *N. piscator* the chromatoid body appeared faint in living cells under ase contrast (Sud, 1955). It was resistant to Bouin fixation. It was not loured by Sudan black nor darkened by silver and long osmication techques, but it stained deeply with iron haematoxylin and with acid fuchsine. concluded that it appeared to arise in the cytoplasm independently of any her inclusion. Similar chromatoid bodies occur in the freshwater turtle,

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Lissemys punctata (Sud, 1956, 1958), and the lizard, *Uromastix hardwicki* (Sud, 1957, 1958).

The fact that the chromatoid body of reptiles appears and disappears during spermatogenesis without making any visible contribution to the final make-up of the spermatozoon remains an enigma. A similar situation exists in mammals, birds, cyclostomes, Crustacea, insects, and arachnids. The accounts given by different authors of the chromatoid body in different species and sometimes even in the same species are diverse. This has led prominent workers, among them Duesberg (1908), the Schreiners (1908), Wilson (1925), and Gresson (1951), to doubt whether all the bodies named as such are of the same nature. To avoid this confusion and a perpetual state of doubt I considered it necessary to undertake a histochemical study of the chromatoid body in different groups. It is hoped that this study combined with electron microscopy may help us to formulate criteria for its identification and to trace its origin and physiological significance. The present communication is the first of the series dealing with this subject.

MATERIAL AND METHODS

Specimens of the grass-snake, *N. natrix* L., were obtained from L. Haig & Co., Beam Brook, Newdigate, Surrey, during different seasons, and kept in a terrarium for periods up to one month. This animal provides ideal materia for the study of the chromatoid body because of the large size of this inclusion in its spermatocytes as well as spermatids. In the spermatid of the snake the chromatoid body appears during the late stages of spermateleosis, which occur in the early winter months when the testis is generally devoid o spermatocytes. The spermatocytes are most abundant during late winter and early spring. So, for the study of the chromatoid body in the spermatocytes as well as spermatids, every test had to be done twice with an interva of a couple of months.

The animals were killed by passing an awl through the brain.

For study of the living material, the testes on removal from the animal were

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FIG. 1 (plate). Sections of the testis of *N. natrix*.

A, formaldehyde/calcium followed by AH test; the labelled cell is a primary spermatocyte showing a AH-negative chromatoid body adjacent to the nucleus (*n*), AH positive nucleolu (*nl*).

B, formaldehyde/calcium followed by AH test; the labelled cell is a late spermatid with AH-positive chromatoid body, nucleus (*n*), and acrosome (*a*).

C, AH test after pyridine extraction; primary spermatocytes at the periphery of the tubul showing AH-positive chromatoid body adjacent to the nucleus (*n*) containing AH-positiv nucleolus (*nl*); chromatoid bodies, not in sharp focus, indicated by arrows.

D, AH test after pyridine extraction; late spermatids showing AH-positive chromatoid bod indicated by arrows; nucleus (*n*) and acrosome (*a*) are also AH-positive.

E, Sanfelice / basic fuchsine; note in the spermatocytes basiphil chromatoid body; nucleu (*n*); basiphil nucleolus (*nl*).

F, Sanfelice / basic fuchsine; late spermatids; note the basiphil chromatoid body an nucleus (*n*); acrosome (*a*).

G, Zenker / crystal violet; two tubules each showing in focus a primary spermatocyte with a basiphil chromatoid body; nucleus (*n*).

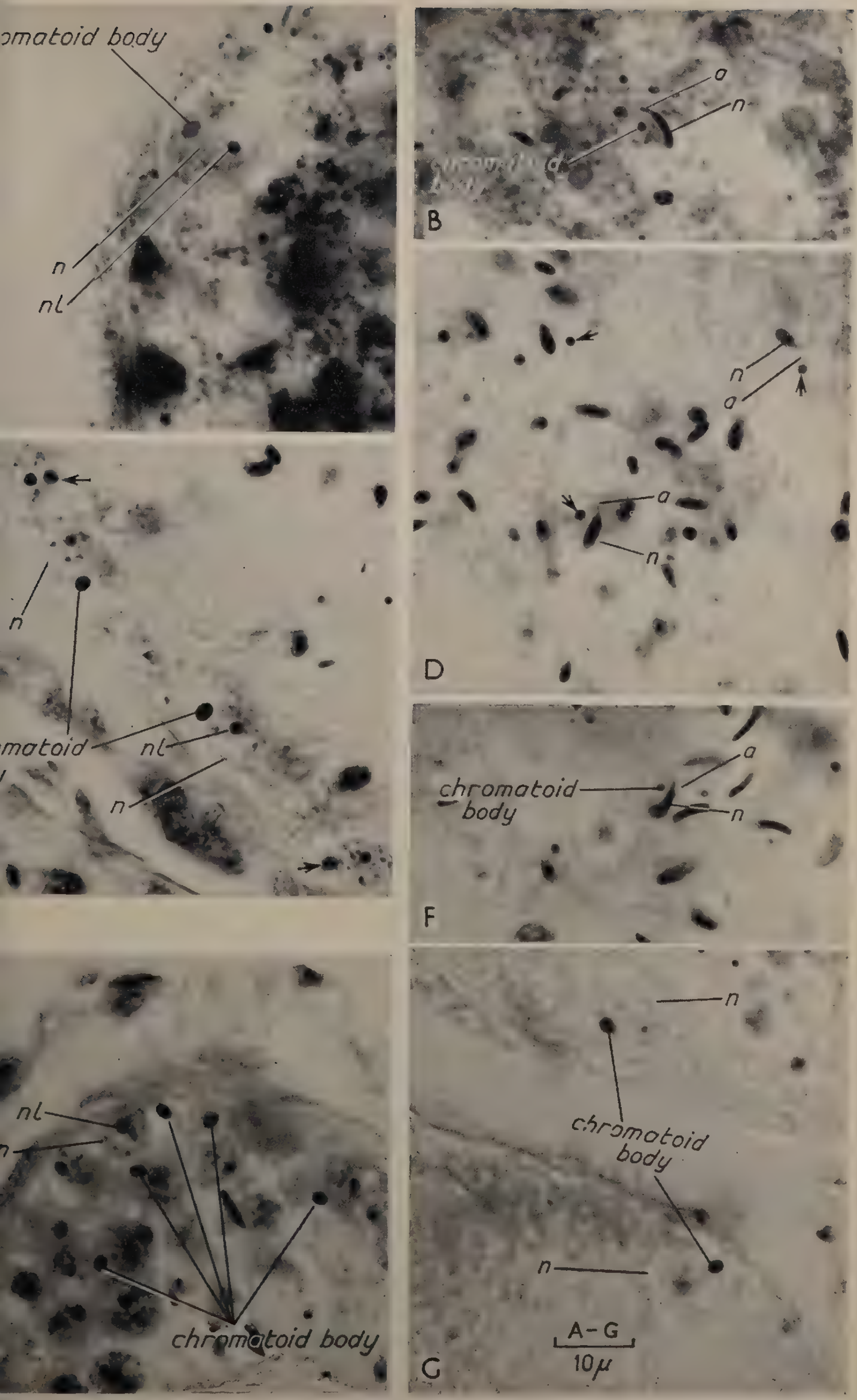
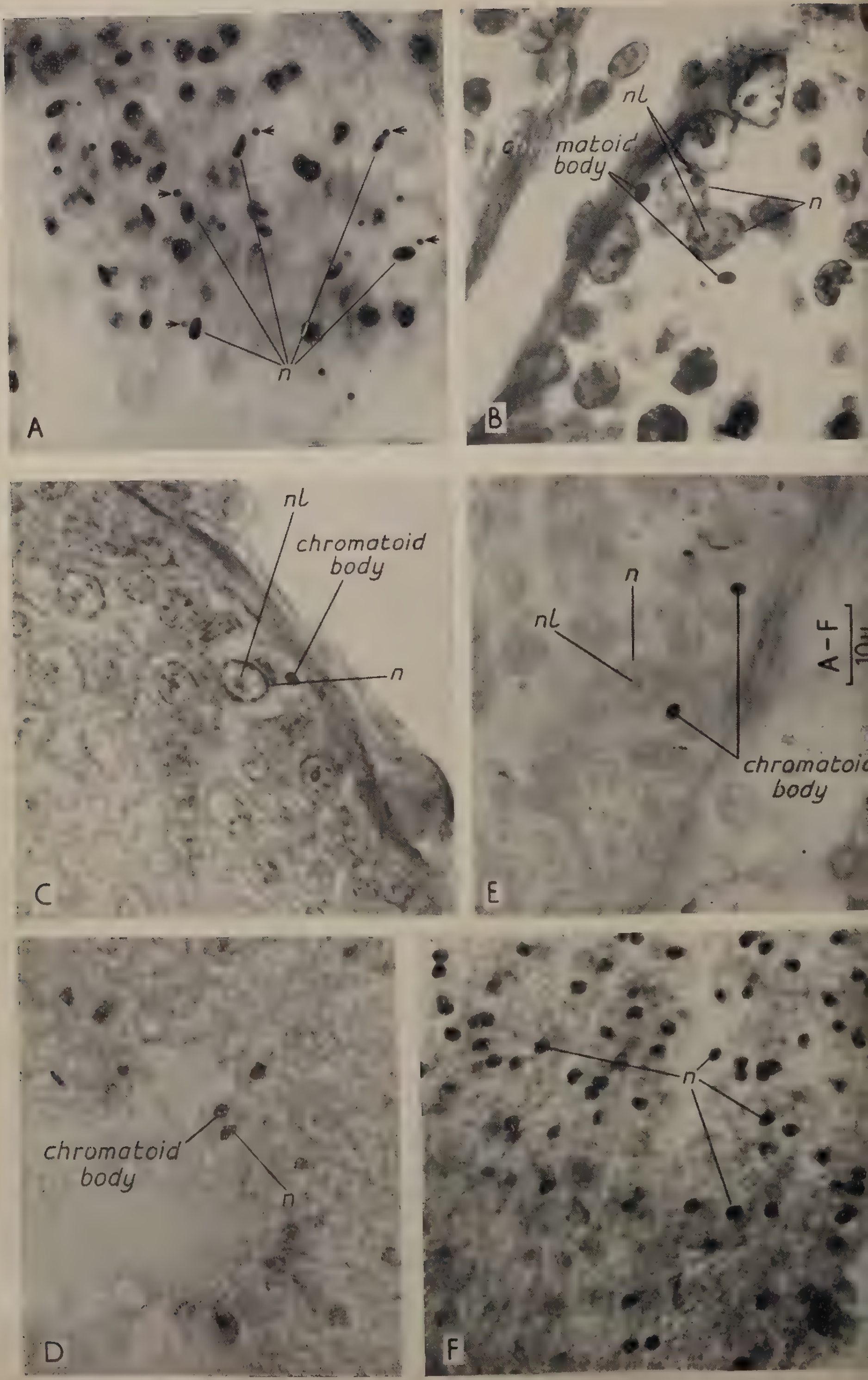

omatoid body
n
nl
B
a
n
chromatoid body
D
n
a
a
n
n
matoid
nl
n
a
chromatoid body
n
F
nl
n
chromatoid body
G
n
chromatoid body
n
A–G
10μ

FIG. 1

B. N. SUD


A
n
B
nl
matoid
body
n
C
nl
chromatoid
body
n
E
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nl
chromatoi
body
A–F
10µ
D
chromatoid
body
n
F
n

FIG 2.

B. N. SUD

once transferred to 0·7% sodium chloride solution, to every 100 ml of
ich 0·2 ml of 10% anhydrous calcium chloride had been added (Baker,
4). A small piece of testis tubule was teased and mounted in the same
ution and studied unstained by phase-contrast microscopy.

Living material was also dyed supravitally with neutral red and with Janus
een. In both cases 0·1 ml of a 0·5% aqueous stock solution of the dye was
uted with 5 ml of the saline solution mentioned in the preceding paragraph.
very small piece of testis tubule was dyed in the diluted solution for 5 to 15
n and later teased and studied by direct microscopy.

For fixation, the testes immediately on removal from the animal were
ided into small pieces by a sharp razor, and the pieces directly transferred
various fixing fluids. The material was embedded in either paraffin wax,
lodion, or gelatine for different histochemical tests, as detailed in the
pendix (p. 56).

For deamination a freshly prepared mixture of equal volumes of 0·1 N
drochloric acid and 0·1 N sodium nitrite was used for 10 min at room
nperature (Chayen & others, 1959). More often a stronger deamination
xture, consisting of 2 volumes of concentrated aqueous sodium nitrite
ution, 1 volume of glacial acetic acid, and 5 volumes of distilled water, was
ed for 12 h at room temperature. This mixture of van Slyke was introduced
o histochemistry by Monné and Slautterback (1950). The preparations
re thoroughly washed after treatment with deaminating fluids. Deaminated
d control preparations were subjected to exactly the same treatment.

OBSERVATIONS

orphological studies

The chromatoid body in *N. natrix* first appears during the growth of the
mary spermatocyte (figs. 1, C, E, G; 2, B, C). It disappears at metaphase of
first maturation division and is again seen in the secondary spermatocyte.
disappears again at the metaphase of the second maturation division and is
t present in the early stages of spermateleosis. It reappears during the
ndensation and elongation of the spermatid nucleus (figs. 1, B, D, F; 2, A, D),
d finally disappears during the ripening of the spermatozoon. It seems
appear and disappear as a whole body, without showing any sign of

---

IG. 2 (plate). A–E, sections of testis, and F, of vas deferens of *N. natrix*.
, Zenker / crystal violet; late spermatids; note the basiphil chromatoid body (indicated by
ows) and nucleus (*n*).
, Zenker / azocarmine; spermatocytes; note the acidophil chromatoid body and nucleolus
; nucleus (*n*).
, Sakaguchi test; the labelled cell is a primary spermatocyte showing a positive chromatoid
ly and nucleolus (*nl*); nucleus (*n*).
, Sakaguchi test; the labelled cell is a late spermatid in the lumen of a tubule; it shows a
itive chromatoid body and nucleus (*n*).
, deamination by van Slyke's reagent / azocarmine; spermatocytes with chromatoid body
wing intense acidophilia and negative nucleolus (*nl*); nucleus (*n*).
, vas deferens after Sakaguchi test; note the intensely positive nuclei (*n*) of spermatozoa.

fragmentation, and does not make any recognizable contribution to the mature spermatozoon.

In the spermatocytes the chromatoid body is of ovoid or subspherical form, measuring up to 3 by $2\frac{1}{4}\ \mu$; in the spermatids it is always spherical and is up to $1{\cdot}8\ \mu$ in diameter. In both spermatocytes and spermatids it is homogeneous.

In the living cell, under phase contrast, it gives a very low phase-change. In the spermatid it gives a slightly higher phase change than in the spermatocyte, though it is much smaller. Thus there is a higher proportion of solid material in the chromatoid body of the spermatid.

Neutral red and Janus green, used supravitally, failed to dye the chromatoid body in both spermatocytes and spermatids.

Histochemical studies

From the tests listed in the appendix on page 56 it is evident that the chromatoid body does not contain lipid. If there were any solid lipid it would have coloured with Sudan black used at 60° C or given a positive Liebermann's reaction. There does not appear to be any masked lipid either, since Sudan black does not colour the chromatoid body in material treated with such strong splitting agents as cadmium chloride (Clayton, 1959) in Aoyama, or chromic acid (Bradbury & Clayton, 1958) in Flemming's fluid, Lewitsky-saline, or Lewitsky's chromium trioxide / formaldehyde. Negative results by silver and long osmication techniques, and by Rawitz's 'inversion staining' technique also suggest the absence of lipid.

The acid haematein test shows a difference of an unexplained kind between the chromatoid body of the spermatocytes and that of the spermatid. The former gives a negative reaction to the AH test itself but a positive reaction to the pyridine extraction test, while the latter gives a positive reaction to both (fig. 1, A–D).

There is no DNA in the chromatoid body. The intense basiphilia of the body (figs. 1, E–G; 2, A) is due mainly to its high content of RNA, but probably also in part to acidic groups in protein.

The chromatoid body stains readily with all the acid dyes tried (fig. 2, B) and the acidophilia indicates the presence of basic proteins. The intense positive Sakaguchi reaction (Baker, 1947) shows the presence of protein-bound arginine (fig. 2, C, D). Free arginine, if present, would probably be removed from the tissue during fixation and dehydration.

After deamination the chromatoid body gives a negative Sakaguchi reaction but there is no change in its intense acidophilia (fig. 2, E). It is clear that the acid dyes must be reacting with basic groups other than —NH_2.

The negative result with the alkaline fast green reagent of Alfert and Geschwind (1953) suggests the absence of histones. Protamines, if present, would be removed during treatment with hot trichloracetic acid for the necessary removal of nucleic acids for this technique (Alfert, 1956). The negative

esult with alkaline fast green suggests that the arginine occurs mostly as a
omponent of protamine rather than histone.

There is little or no tyrosine in the chromatoid body. Tests for carbo-
ydrates, alkaline phosphatase, and calcium also give negative results.

The evidence suggests that the chromatoid body consists mainly of RNA
nd of proteins with abundant acidic and basic groups.

DISCUSSION

Protamines consisting predominantly of arginine residues replace the
stones of chromatin during spermatogenesis. Fig. 2, F shows the strong
eaction of the nuclei of the ripe spermatoza of *Natrix* with the Sakaguchi
eaction. It appears that in the salmon this change takes place rather abruptly
a late stage of spermateleosis, when the nucleus begins to elongate (Alfert,
956).

In *Natrix* the chromatoid body reappears during spermateleosis at the
me when the characteristic change in the nucleoprotein of the spermatid
robably takes place. Further, the chromatoid body in *Natrix* is characterized
y the presence of basic proteins rich in arginine and by the absence of his-
nes. Thus the protein constituents of the sperm nucleus and of the chroma-
id body are remarkably similar.

The histochemical reactions of the chromatoid body are very similar to
ose of the nucleolus (see appendix). The abundance of RNA and proteins
the chromatoid body may perhaps indicate that it acts as a centre for protein
nthesis. It may be tentatively suggested that it is the function of this body
provide basic proteins for the final maturation of the chromatin in the
ucleus of late spermatids.

I am grateful to Dr. J. R. Baker, F.R.S., for teaching me histochemistry
d supervising this investigation, and to Professor Sir A. C. Hardy, F.R.S.,
r providing me research facilities in his Department. I wish to thank Mrs.
M. Luke and Miss Elizabeth Collins for their valuable technical help.
am greatly indebted to the Vice-Chancellor, University of the Panjab
ndia), for the grant of study-leave.

APPENDIX

A summary of the histochemistry of the chromatoid body and nucleolus in Natrix natrix

Tests applied					*Results obtained*		
					Chromatoid body		
Test	*Fixation*	*Embedding medium*	*Thickness of section in μ*	*Reference*	*In spermatocytes*	*In spermatids*	*Nucleolus in spermatocytes*
Natural colour					Colourless		
Neutral red	living tissue				O	O	O
Janus green	living tissue				O	O	O
Sudan black (RT; 60° C)	F/S+PC	G	10	Baker, 1944, 1949	O	O	O
Sudan black (RT; 60° C)	F/Ca+PC	G	10	Baker, 1946	O	O	O
Sudan black (RT; 60° C)	WB+PE	G	10	Baker, 1946	O	O	O
Sudan black (RT; 60° C)	Aoy+PC	G	10	Baker (unpublished)	O	O	O
Sudan black (RT; 60° C)	Cr/F+PC	G	10	Lewitsky, 1931	O	O	O
Sudan black (RT; 60° C)	Flm+PC	G	10	Bradbury & Clayton, 1958	O	O	O
Sudan black (RT; 60° C)	LS	G	10	Baker, 1956*a*	O	O	O
Acid haematein	F/Ca+PC	G	10	Baker, 1946	O	+++	+ to +++
Acid haematein with pyridine extraction	WB+PE	G	10	Baker, 1946	+++	+++	O to ++
Acid haematein	Aoy+PC	G	10	Baker (unpublished)	O	+++	O
Acid haematein	Cr/F+PC	G	10	Chayen & others, 1959	+++	+++	+++
Acid haematein	Flm+PC	G	10	Bradbury & Clayton, 1958	O	+++	O to +++
Liebermann	F/Ca; F/Ca+PC	G	10	Lison, 1953	O	O	O
Silver impregnation	Aoy	P	6	Aoyama, 1929	O	O	O
Weigl (Mann–Kopsch)	Mann PO	P	2	Weigl, 1910	O	O	O
Kolatchev	Champy PO	P	2	Kolatchev, 1916	O	O	O
Baker (Hermann–Kopsch)	Hermann PO	P	2	Baker, 1957	O	O	O
Rawitz	Flm	P	6	Baker, 1959; Przełęcka, 1959	O	O	O
Toluidine blue for metachromasy	Z	P	10	Baker (unpublished)	O	O	O
Periodic acid / Schiff	Z	P	6	McManus, 1948; Pearse, 1954	O	O	O
Feulgen	Z; Cl	P	8; 16	Feulgen & Rossenbeck, 1924	+++	+++	++
Pyronin / methyl green	Z 3 h	P	7; 16	Jordan & Baker, 1955	++	++	+
P/MG after trichloracetic acid	Z 3 h	P	7; 16	Pearse, 1954	O	O	O
P/MG after ribonuclease	Z 3 h	P	7; 16	Bradbury, 1956	+++	+++	+++
Basic fuchsine (0·5% aq., RT) for basiphilia	S; Flm	P	6	—	+++	+++	+++
Safranine (0·5% aq., RT) for basiphilia	Z 3 h; Flm	P	6	—	+++	+++	+++

Methyl green (1% aq. RT) for basiphilia	Z 3 h	P	6	—	O	O	O
Methyl green (1% aq. 50° C) for basiphilia	Z 3 h	P	6	—	+	+	+
Newton's crystal violet	Z 3 h	P	14	Baker & Jordan, 1953	+++	+++	O to +
Newton's crystal violet	S	P	14	Baker & Jordan, 1953	++	++	+ to ++
Nissl stain (cresyl violet)	Z 3 h	P	6	Fernstrom, 1958	+++	+++	+++
Iron haematoxylin	Z 3 h; Flm	P	6	—	+++	+++	+++
Acid fuchsine (1% aq., RT) for acidophilia	Z 3 h	P	8	—	+++	+++	+++
Eosin (1% aq., RT) for acidophila	Z 3 h	P	6	—	++	++	++
Metzner	Alt; Flm; H+PC	P	6; 3	Metzner & Krause, 1928	+++	+++	+++
Azocarmine (2% aq., acidified, 50° C)	Z 3 h; Cl	P	6	Gatenby & Beams, 1950	+++	+++	+++
Deamination by van Slyke reagent / azocarmine (2% aq., acidified, 50° C)	Z 3 h; Cl	P	6	Monné & Slautterback, 1950	+++	+++	O
Xylidine red (0·1% in 1% acetic acid, RT)	Z 3 h; Cl	P	6	——	++	++	++
Deamination by van Slyke reagent / xylidine (0·1% in 1% acetic acid, RT)	Z 3 h; Cl	P	6	Monné & Slautterback, 1950	++	++	O
Sakaguchi	Z	P	10	Baker, 1947	+++	+++	+ to ++
Deamination by van Slyke reagent / Sakaguchi	Z	P	10	Monné & Slautterback, 1950; Baker, 1947	O	O	O
Hg/nitrite	F/S	C	24	Baker, 1956*b*	O	O	O
Coupled tetrazonium reaction	F/S	P	16	Danielli, 1947, 1950; Pearse, 1954	O	O	O
Alkaline fast green method	F	P	16	Alfert & Geschwind, 1953	O	O	O
Gomori's for alkaline phosphatase	Alc/Acet	P	8	Gomori, 1952	O	O	O
Cobalt method for calcium	F/M/P	P	10	As in alkaline phosphatase test used by Danielli (1946)	O	O	O

KEY: Alc/Acet = absolute alcohol and acetone in equal volumes; Alt = Altmann's fluid; Aoy = Aoyama's fluid; aq. = aqueous; C = collodion; Cl = Clarke's (Carnoy's) fluid; Cr/F = chromium trioxide/formaldehyde; F = formaldehyde; F/Ca = formaldehyde/calcium; Flm = Flemming's fluid; F/M/P = formaldehyde/methanol/pyridine; F/S = formaldehyde/saline; G = gelatine; H = Helly's fluid; LS = Lewitsky-saline; P = paraffin; PC = postchromed; PO = postosmicated; RT = room temperature; S = Sanfelice's fluid; WB+PE = weak Bouin+pyridine extraction; Z = Zenker's fluid; 3 h = 3 hours; +++ = strong reaction; ++ = moderate reaction; + = weak reaction; O = negative reaction.

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The Chemical and Physical Nature of the Visible Regions of Mouse Ascites Tumour Cells, and of the Cells of Solid Tumours grown from them

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With one plate (fig. 2)

SUMMARY

A new technique was used to embed separate mouse ascites cells of 7 different strains in a supporting medium so that they could be sectioned, and a number of histochemical tests were carried out on this material. The results of these were in good general agreement with the findings of Love & others (1953, 1956), who investigated the histochemical properties of dried smear preparations of ascites cells of very similar strains; but certain important differences were found, notably the present investigation revealed the presence of phospholipid in almost all the lipid droplets in the cytoplasm. No important differences were found in the histochemical properties of the cells of the 7 different strains examined, nor between these and the cells of subcutaneous solid tumours grown from them.

The solid content of the cytoplasm, nuclear sap, and nucleoli of the living cells of 6 of these 7 strains were measured by immersion refractometry and interference microscopy. The mean total solids in the cytoplasm were, in all strains, near to 14% w/v and the concentration in the nuclear sap was usually a little lower than this. The total solid in the nucleoli was approximately 50% w/v. Intact living cells were also isolated from the solid tumours by means of a special 'homogenizer', and the total solid in their cytoplasm was in all cases identical with that of the ascites cells from which they were derived.

From these data it seems probable that the chemical and physical characteristics of many different strains of ascites cells, including those of carcinomatous, sarcomatous, and leukaemic origin, do not differ markedly from one another; and are also probably not very different from those of the cells of the solid tumours from which they were originally derived.

INTRODUCTION

AN extensive histochemical investigation of the visible constituents in mouse ascites tumour cells of 10 different strains was undertaken by Dr. Love & his colleagues in 1953 and 1956. This yielded much valuable information but was open to one serious criticism pointed out by the authors themselves (Love & others, 1953); namely, that all their tests were carried out on dried 'smear' preparations of the separate cells, whereas some of the histochemical tests they used (notably the acid haematein test of Baker (1946) for phospholipid) were never intended to be used on anything other than sections of compact tissues. It therefore seemed desirable to devise some means whereby the ascites cells could be embedded in a suitable medium

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so that they could be sectioned and treated in exactly the same way as solid tissues; and that their more important histochemical properties should be reinvestigated. This was made possible by the development of a new embedding technique (described below on p. 61) which involved suspending the living cells in a concentrated solution of isotonic bovine plasma albumin before they were fixed, and thus forming a material that could be sectioned like a tissue.

These investigations were accompanied by quantitative investigations by interference microscopy of suspensions of the living ascites cells. The method of immersion refractometry used by Barer and Ross in 1952 was employed to determine the total solids in the cytoplasm and nuclear sap of these cells; and in some cases it was also possible to obtain measurements of the refractive indices of certain of the cell inclusions identified by the histochemical methods, and to draw definite conclusions from these measurements.

Most of the strains of ascites cells investigated were also grown as solid tumours by the subcutaneous injection of a suspension of the ascites cells into the host mice, and these, too, were investigated histochemically. It was also possible to separate the individual living cells in these solid tumours by means of a special 'homogenizer', and the same technique of immersion refractometry was used to obtain measurements of their cytoplasmic solid content.

MATERIAL

The material investigated consisted of ascites tumour cells of 7 recognized strains that were being propagated in the body cavities of mice at the Chester Beatty Research Institute in 1959. Three of these strains were derived from carcinomas, 2 from sarcomas, and 2 were leukaemic in origin. A summary of their known characteristics is given in Appendix 1 (p. 73).

Two-thirds of all the individual transplants investigated were between 5 and 8 days old, and the ages of the remainder ranged from 3 to 15 days. The initial histochemical tests were all done on cells from 7-day-old transplants, but when these were repeated on cells from transplants of different ages, they yielded identical results. The physical properties of the cells, too, were never found to vary with the age of the transplant (within the range investigated) in any recognizable way.

The 8-day-old and 12-day-old solid tumours were formed by the subcutaneous injection of suspensions of ascites cells from 4 of the 7 strains investigated. Macroscopically, they resembled solid tumours not of ascites origin, and microscopically they were composed mostly of cells similar in size and appearance to the ascites cells grown in the body-cavity, but less regular in shape, and bound together with connective tissue and blood-vessels just as many other solid tumour tissues are. Sections of these were mounted with similarly fixed separate ascites cells of the same strain from the body-cavity, side by side on the same slide, and subjected to identical treatment in all the histochemical investigations. In all the preparations so treated the nuclear material, cytoplasm, and inclusions of the cells in the solid tumours were found to have the same histochemical properties as those of the separate ascites cells grown in the body-cavity.

INVESTIGATION OF THE CHEMICAL NATURE OF THE DIFFERENT REGIONS IN THE ASCITES TUMOUR CELLS

Embedding techniques for the separate ascites cells

The method of mounting the separate ascites cells from the body-cavity was an extremely simple one. It consisted of surrounding the individual cells with a matrix of homogeneous protein, which, after fixation, formed an artificial 'tissue' enclosing the cells.

A few drops of the ascites fluid containing suspended cells were withdrawn from the body-cavity of the animal with a syringe and immediately mixed with approximately twice the volume of a concentrated solution of isotonic bovine plasma albumin. The product used was the bovine plasma albumin, fraction V, manufactured by the Armour Laboratories, Kankakee, Illinois, U.S.A. (and also obtainable from the Armour Laboratories, Eastbourne, England). The dried powder contains about 0·75% of salt, and a 40% solution dissolved in 0·5% NaCl is isotonic with mammalian body-fluids (see Ross, 1961); and the ascites fluid was added to this. The resulting mixture of suspended cells, ascites fluid, and saline/protein worked very satisfactorily as a supporting medium if it had a refractive index higher than about 1·375: in suspensions where the protein was more dilute than this the ascites cells usually no longer retained their spherical shape after being fixed, although they reacted in exactly the same way to the histochemical tests (compare fig. 2, E and G).

With many of the fixatives that contain a protein precipitant (such as the Zenker's fluid, which was used in the investigation of nucleic acids and proteins), it was only necessary to let a drop of this mixture fall from a fine pipette into the fixing fluid, whereupon the protein immediately coagulated into a compact mass (frequently mushroom-shaped) about 1 mm in diameter. This was then embedded in paraffin wax and sectioned in exactly the same way as an ordinary piece of tissue. A section so prepared and treated for the Feulgen reaction is illustrated in fig. 2 A.

When non-protein-precipitant fixatives were used, however (such as the formaldehyde-calcium used in investigating lipid inclusions), a drop of the mixture added to the fixative solution simply dispersed or formed a 'mud' at the bottom of the solution, which was impossible to handle. In these cases a method developed by Baker (1943) was used to keep the cells together. A capsule to contain the suspension was first prepared from an easily cut, porous material which had been previously soaked in isotonic saline, by excavating a depression in one side of a small cube of the material about 3 mm across. A drop of the cell suspension in protein was placed in this cavity and covered with a 'lid' of the same material which was then tied on with a silk thread (see fig. 1). The complete capsule was then placed in the fixative and subsequently embedded in gelatine, from which frozen sections were cut. The porous material forming the capsule was, in a few cases, mammalian liver that had been fixed in picric acid, but for the most part the milk curd, fixed in alcohol and extracted with ether-alcohol, described by Baker (1943), was employed. Both materials were equally satisfactory as supporting media, but the milk curd had the advantage that it was itself entirely free of cells, so that any cells visible in preparations where it was used were certain to belong to the suspension contained within it. The ascites cells were, however, so different morphologically from liver cells that, with this material, the chances of their being confused with them were negligible. Cells prepared in this manner can be regarded as subject to a form of 'triple embedding', as they were first surrounded by protein, then enclosed by the capsule material, and finally embedded in gelatine.

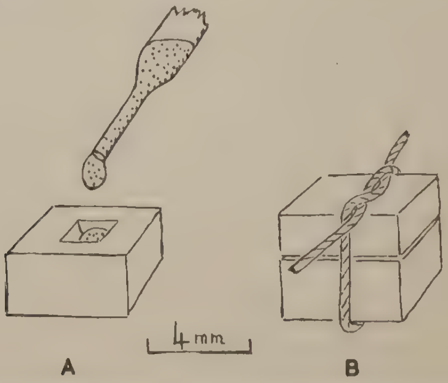

FIG. 1. Drawings showing the method of enclosing a supension of separate acites cells in a block of supporting material before fixation. A, a cavity in a block of the supporting material impregnated with isotonic saline being filled from a pipette with a suspension of the living cells in isotonic bovine plasma albumin. B, the same block covered with a lid of the same material tied on with a fine silk thread, ready to be placed in the fixative solution. (The supporting material usually used here was milk-curd fixed in alcohol and washed with alcohol/ether before impregnation with saline.)

Results of the histochemical investigations

The histochemical investigations made on sections prepared in the manner described enabled all the regions of the ascites cells that are visible in living preparations with phase-contrast or interference microscopy to be separately identified: and these results are summarized in Appendix 2 (p. 74). In general, they confirm the findings of Love and his associates (1953, 1956), but with several important differences discussed below. The present investigation also agreed with that of these workers in revealing no differences of any importance in the histochemical nature of the ascites cells of the different strains examined.

Nuclear material. The Feulgen reaction showed nothing remarkable in the distribution of DNA. This is confined to the chromatin, in complete agreement with the findings of all previous workers. It was in respect to the composition of the nucleoli that certain differences were found from the findings of Dr. Love and his associates. They, in their smear preparations, were unable to demonstrate the presence of RNA in this position, while the present tests with the pyronine and methyl-green technique described by Jordan and Baker (1955) indicated that it was almost always present (see fig. 2, B). Control sections treated with freshly prepared rhibonuclease in the manner described by Bradbury (1956) showed that occasionally some other basiphil material was also present in some of the nucleoli. Love and his colleagues did not find arginine in this position, while the Sakaguchi reaction carried out on the sections in the present investigation gave an appreciably stronger reaction in the nucleoli than in the cytoplasm (see fig. 2, I).

Cytoplasmic RNA. Basic fuchsine and pyronine staining indicated the presence of basiphil material evenly distributed over most of the cytoplasm except in the idiozome, which is a special region usually associated with an indentation in the nucleus that is often visible in the ascites cells of carcinomatous and sarcomatous origin, and which is probably acidophil (see fig. 2, B). Control sections treated with freshly prepared rhibonuclease demonstrated that the general basiphil area of the cytoplasm contained RNA. This is in complete accord with the findings of Love and his associates, who refer to the idiozome region in their papers as the 'cytoplasmic nuclear hof'; but the term 'idiozome', first used by Meves in 1896, possesses less ambiguity. This region appeared to be absent in nearly all the ascites cell of leukaemic origin (in the EL4 and C+leukaemia strains) that were investigated.

Cytoplasmic lipid droplets. It was in regard to the nature of the lipid droplets in the cytoplasm that the results of the present investigation and those of Love and his associates differ most strikingly, although a great many of their findings were confirmed also. In the living ascites cells of all the strains investigated these lipid droplets can be seen to be of two kinds: large lipid droplets 0·5 μ to 3·5 μ in diameter, and small lipid droplets mostly less than 0·5 μ in diameter which Love and his associates call lipochondria. These workers demonstrated that many of the larger droplets were of a composite nature, with an internal part consisting of a neutral lipid surrounded by an external shell of a different lipid, the precise nature of which they were not able to determine. In their tests, these larger globules consistently reacted negatively to Baker's acid haematein in test applied to their smear preparations. There is some uncertainty, however, in the results they obtained in applying this test to the smaller lipid droplets, since in their first paper (1953), which was confined to Ehrlich ascites cells, they reported a faint positive reaction, while in their second paper (1956) a fuller investigation on a wider variety of strains led them to change their minds and to state that this test was consistently negative.

The present investigation on frozen sections of ascites cells showed quite beyond dispute a positive reaction to Baker's acid haematein test with both the large and the small lipid droplets, and a negative result obtained after pyridine extraction indicated that they both contained phospholipid (see fig. 2, E–G). In certain instances also, convincing supporting evidence for the presence of phospholipid in the larger droplets was obtained from refractive index measurements made on them in the living cells. This will be described below (p. 69).

With the larger lipid droplets, the acid-haematein-positive region was the external shell, which was found to be always present but varied somewhat in thickness in different strains of ascites cells, being thickest in cells of the Krebs and Crocker strains, where the shells frequently presented the appearance of thick dark rings (fig. 2, G). The same droplets treated with Sudan black were stained all through. This indicated that the central part of the droplets were also lipid although of a different kind. This was strikingly demonstrated when the acid-haematein preparations were subsequently stained with Sudan IV: a technique suggested by Dr. S. Bradbury. The large lipid droplets were then frequently found to have a bright red centre surrounded by a black outer shell (fig. 2, G). Cain's Nile blue test (1947, 1948) showed up this centre as slightly pink in some of the larger droplets, although this reaction was usually masked by the intensely blue staining of the externum. This indicated that the internal part was probably a neutral lipid. Just occasionally in the Sudan black preparations a few large lipid droplets in some of the cells presented a ring-like appearance with an unstained centre. These probably were globules containing only phospholipid surrounding a watery vacuole.

The smaller lipid droplets all reacted positively to the acid haematein test,

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FIG. 2 (plate). A–I, photomicrographs, under a 2-mm objective, of sections of 3 different strains of mouse ascites cells (embedded in bovine plasma albumin) and of solid tumour cells grown from them, subjected to different histochemical reagents.

A, 7-day Ehrlich ascites. Feulgen.

B, 8-day Landschütz ascites. Pyronine / methyl green, showing RNA distribution in the nucleoli and cytoplasm. *i*, idiozome region.

C, 7-day Ehrlich ascites. Basic fuchsine.

D, 7-day Ehrlich solid tumour cells. Basic fuchsine.

E, 7-day Landschütz ascites. Acid haematein, *el*, externum of large lipid droplets. *s*, small lipid droplets.

F, 7-day Landschütz solid tumour cells. Acid haematein. *el* and *s*, as in E.

G, 7-day Crocker ascites. Acid haematein and Sudan IV. *l*, large composite lipid droplets. *s*, small lipid droplets.

H, 8-day Ehrlich ascites. Metzner. 2-μ section showing mitochondria.

I, 7-day Ehrlich ascites. Sakaguchi. *an*, arginine especially concentrated in the nucleoli.

J–K, negative prints from colour photomicrographs of living Landshütz ascites cells, mounted in isotonic bovine plasma albumin, under a Smith interference microscope with a 2-mm shearing objective.

J, a group of cells from a 6-day-old transplant, with conspicuous lipid inclusions, mounted in a medium with a refractive index of 1·356. The cytoplasm of most of the cells matches the background intensity, and the phase changes given by the individual lipid droplets can be measured.

K, cells from a 9-day-old transplant mounted in a medium with a refractive index of 1·360. In all the resting cells the nuclear sap matches the cytoplasm and background in intensity, and the phase-changes given by the nucleoli can be measured. *d*, a dividing cell in telophase.

20 μ Scale; for A–H as indicated in B, and for J and K as indicated in K.

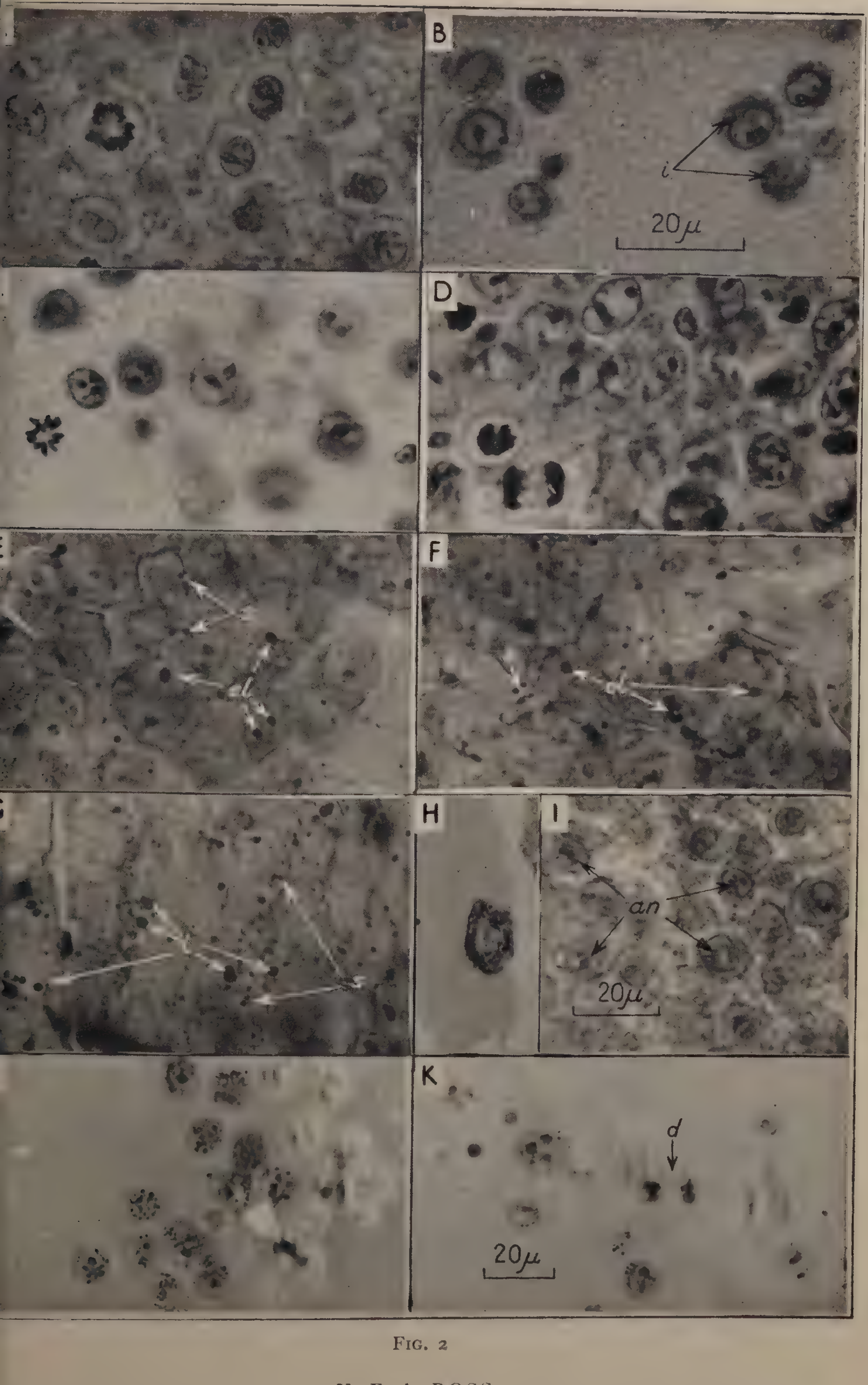

FIG. 2

K. F. A. ROSS

and therefore contained some phospholipid. They also gave a weak positive reaction to the PAS test, which probably indicated the presence of other substances as well. Lack of time prevented further investigation of their nature, but there is no evidence to suggest that the findings of Love and his associates, which indicated the presence of glycolipid, glycoprotein, or fatty acids in these small droplets, were incorrect.

Liebermann's test for cholesterol was consistently negative in both the large and small lipid droplets, but this does not exclude the possibility of there being small amounts of this substance in both or either of these droplets, because the test is not very sensitive (Schultz, 1924, 1925).

The small droplets in the living cells mounted in saline were coloured strongly with neutral red, but the large lipid droplets were only occasionally coloured strongly. Most of them remained almost colourless, but a faintly coloured region at their edges was frequently visible, especially in the ascites cells of the Crocker and Krebs strains.

Mitochondria. In most of the preparations described the mitochondria were destroyed by fixation; but with material fixed in Altmann's or in Helly's fluid small bodies in the form of spheres or very short rods were visible after the preparations had been stained by Metzner's method, scattered evenly through the cytoplasm. These were barely visible in unstained living preparations, but are presumably the same bodies as stain with Janus green (see fig. 2, H).

INVESTIGATION OF THE PHYSICAL NATURE OF THE DIFFERENT REGIONS IN THE LIVING ASCITES TUMOUR CELLS

After the chemical nature of the visibly different regions in the ascites cells had been identified, as far as possible, it was clearly desirable to try to obtain quantitative estimates of the concentrations of these substances in the living cells by refractometry and interference microscopy. This was only possible in a limited number of instances, because it could not be done with any of the very irregularly distributed nuclear material such as the chromatin, nor with the smaller cytoplasmic inclusions, since it was impossible to estimate their thickness with any accuracy. Measurements could, however, be obtained for the larger regular regions, and reasonably accurate measurements were obtained for the refractive indices of the cytoplasm, the nuclear sap, the nucleoli, and the larger lipid droplets in the cytoplasm.

Refractometric techniques

The methods used were those of the immersion refractometry of living cells developed by Barer and Ross in 1952, and of immersion refractometry combined with interference microscopy first used by Ross in 1954; both of which are described in some detail by Ross (1961 and elsewhere). For measuring the refractive index of the cytoplasm, the living cells were immersed in a range of isotonic saline/protein solutions of different refractive indices (the protein being the same Armour's bovine plasma albumin described above on p. 61) until one was found in which the cytoplasm appeared to match the background field in intensity or colour when examined under an interference microscope. If, as frequently happened, the refractive index of the nuclear sap was the same as that of the cytoplasm, the same technique could be used to measure this. From these values a close approximation of the total solid and water content of these regions could be calculated by assuming that the mean specific refraction increment of the dispersed solids in these regions was 0·0018 (see Davies & others, 1954,; Barer & Joseph, 1954).

In preparations of this kind (e.g. as in fig. 2, J), with the refractive index of the cytoplasm the same as that of the mounting medium, the refractive indices of the larger and more regularly shaped inclusions could be obtained by measuring their diameter and the phase change in light of known wavelength passing through them (Ross, 1954; Ross & Chou, 1957). The refractive index of the inclusion n_i was obtained from the formula

$$n_i = \phi \times \frac{\lambda}{t} + n_m,$$

where ϕ is the phase change measured through the inclusion (in fractions of a wavelength), t is the measured thickness of the inclusion (in μ), λ is the wavelength of light used (in μ), and n_m is the refractive index of the mounting medium and the cytoplasm. The accuracy of the method is limited, not by the accuracy with which it is possible to measure phase changes with the interference microscope, but by the accuracy with which the thickness of an object can be measured in the visible spectrum. Even with objectives of the highest possible numerical aperture, an error of up to 0·4 μ is possible. The upper and lower limits of error in any refractive index measurement made in this way can therefore be defined by assuming errors of $\pm 0{\cdot}2\ \mu$ in the thickness measurements, and calculating for these values as well.

The total solids in the cytoplasm

The mean refractive indices of the cytoplasm in 6 of the 7 strains of ascites cells investigated histochemically, and the upper and lower limits of cytoplasmic refractive index found in most of these strains, is shown in table 1, with the per cent total solids that these values represent. In addition, the table shows the refractive indices of the cytoplasm of the cells of the solid tumours grown subcutaneously from 4 of these strains. The separation of these cells in an intact condition was made possible by means of a simple but extremely effective glass 'cell homogenizer' made by Mr. Joseph Kopp of 70–73, 35th Road, Jackson Heights 72, Long Island, U.S.A. This consisted of a ground glass plunger about 1 cm in diameter, working in a ground-glass test-tube-shaped cylinder very carefully made so that the clearance between the cylinder and plunger was between 40 and 60 μ. When a small piece of solid tissue was placed in the cylinder together with about 1 ml. of saline, and the plunger was pushed down and withdrawn once only, it was almost completely broken up into a murky suspension which contained a remarkably

TABLE 1

Refractive indices and equivalent per cent total solids of the cytoplasm of the living ascites cells and of the cells of the solid tumours grown from them

(The values for the per cent solid content of the cytoplasm are immediately below the equivalent refractive index values)

	Ascites cells			*Solid tumour cells (1 transplant examined in each case)*	
Strain of tumour	*Number of transplants examined*	*Mean refractive index and solid content of cytoplasm*	*Lower and upper limits of refractive index and solid content of cytoplasm*	*Mean refractive index and solid content of cytoplasm*	*Lower and upper limits of refractive index and solid content of cytoplasm*
Ehrlich	4	1·357 13·0%	1·353–1·363 10·5%–16·0%	1·358 13·5%	upper limit 1·363 only 16·0%
Landschütz	4	1·359 14·0%	not determined	not examined	
Krebs 2	5	1·358 13·5%	upper limit 1·362 15·5%	not examined	
Sarcoma 1	6	1·361 15·0%	1·356–1·368 12·0%–19·0%	1·359 14·0%	not determined
Crocker	4	1·358 13·5%	1·350–1·363 9·0%–16·0%	1·355 12·0%	1·349–1·363 8·5%–18·0%
EL4	3	1·360 14·5%	1·356–1·365 12·00%–17·5%	1·360 14·5%	1·356–1·366 12·0%–18·0%

high yield of separate, intact cells. This suspension was then mixed with a bovine plasma albumin solution for refractometry of the cytoplasm. (This homogenizer has recently been found to be equally satisfactory for isolating intact living cells from quite a number of normal mammalian tissues, and it may prove to be a valuable research tool.) The cells separated from the solid tumours in this way were not quite as regularly spherical as the ascites cells from the body-cavity, but they were indistinguishable from them in internal structure. From table 1 it can be seen that their cytoplasmic refractive indices were in almost every case very close indeed to those of the loose ascites cells of the same strain. These results show also that, although there is some indication that the cytoplasmic solid content differs characteristically in different strains of ascites, these differences are small and probably not very important.

The total solids in the nuclear sap

In all the strains of ascites cells examined, the refractive index of the nuclear sap was either the same as that of the cytoplasm or a little lower. In the Ehrlich and the EL4 strains, the refractive index of the nuclear sap was most frequently the same as that of the cytoplasm, while in the Krebs and Crocker strains it was more often slightly lower. In all the strains, a considerable number of cells could be found with refractive indices the same as the cytoplasm; and in these cells it was possible to measure the refractive indices of the nucleoli (see fig. 2, K). It may therefore be concluded that the solid concentration of the nuclear sap was either the same as that of the cytoplasm or only a very few per cent lower. It was never recorded as being higher.

The solid content of the nucleoli

The nucleoli of the ascites cells were rather irregular in shape, and often consisted of compact masses joined together by more diffuse regions giving the same staining reactions (see fig. 2, B–D). It was therefore not possible to estimate their thickness in the direction of the optical axis of the microscope (the direction in which phase changes were measured) with very great accuracy by measuring their dimensions in the plane of the microscope field. Only the denser, more compact, regions, where these dimensions were probably nearly the same, were measured in order to obtain values for their refractive index. Table 2 shows the result of 8 such measurements of nucleoli in the cells of the 6 different strains examined. It will be seen that the values obtained are in fact all fairly close to each other and indicate solid contents ranging from 39·5 to 66%. The histochemical findings indicate that most of this solid matter must be RNA.

The refractive indices of the large lipid droplets

Table 3 shows the refractive indices of 20 spherical lipid droplets more than 1 μ in diameter from 5 of the 6 strains examined, and a single, less accurate measurement on a smaller lipid droplet from the EL4 (leukaemic) strain, where, in general, the droplets are smaller. The upper and lower limits of accuracy of all these refractive index measurements calculated on the basis of an error of ±0·2 μ in the measurement of their thickness are given in the final column.

By far the majority of the values obtained are similar to the known values of many pure lipid substances (quoted, for example, in Hodgman, 1945): but 4 of the droplets from the Krebs and Crocker strains (indicated by asterisks in table 4) had refractive indices which, even allowing for the maximum errors in measurement, were lower than that of any pure lipid substance. The only justifiable conclusion that one can draw is the same as that drawn by Ross and Chou in 1957 in respect of rather similar lipid droplets in the nerve-cells of *Helix aspersa*; namely, that these droplets must consist, in the main of a hydrophil lipid substance intimately associated with water molecules. Apart from cholesterol, almost the only lipid substances that are hydrophil in this way are the phospholipids where the phosphoric acid/choline radicle tends to orientate itself towards an aqueous phase, so that the molecules, in association with one another, form bimolecular laminae (Schmidt, 1939). Therefore it seems likely that these particular droplets contained a high proportion of phospholipid. The higher refractive indices of the remaining lipid droplets does not exclude the possibility of their containing phospholipid also, but in smaller amounts. Thus the measurements of the physical properties of these lipid droplets seem to support the histochemical findings.

TABLE 2

The refractive indices of the nucleoli in ascites cells of 6 different strains measured in cells where the refractive index of the nuclear sap was the same as that of the cytoplasm, and their equivalent per cent solid content

(*The values are based on the assumption that the dimensions of the nucleoli measured at right angles to the optical axis of the microscope were the same as those along this axis, and this may not always have been quite true.*)

Strain of ascites	*Refractive index of nuclear sap and cytoplasm*	*Measured width of nucleolus region*	*Refractive index of nucleolus region*	*Equivalent % total solids in nucleolus region*
		(μ)		% w/v
Ehrlich	1·357	2·4	1·413	44·0
,,	1·355	1·8	1·453	66·0
Landschütz	1·360	2·4	1·419	47·0
Krebs	1·358	1·8	1·430	53·5
Sarcoma 1	1·360	3·0	1·405	39·5
Crocker	1·359	2·4	1·424	50·0
,,	1·359	1·7	1·425	50·5
EL4	1·361	1·6	1·418	46·5

A further point of considerable interest is provided by a study of the published electron micrographs of ascites cells, for example, those of Selby and others (1956), of Ehrlich ascites cells. These quite frequently show fragments

TABLE 3

Phase changes, measured through 21 large lipid droplets in 6 different strains of ascites cells, together with their thickness and the values for refractive indices calculated from these data

The final column gives the upper and lower limits of the refractive indices of these droplets, assuming an error of ±0·2 μ in the measurement of their thickness

Strain of ascites	*Refractive index of cytoplasm*	*Measured phase change (degrees)*	*Measured thickness* (μ)	*Refractive index of droplet*	*Upper and lower limits of refractive index, assuming an error of ±0·2μ in thickness measure*
Ehrlich	1·361	188	1·6	1·539	1·518–1·563
,,	1·357	218	2·4	1·494	1·483–1·507
Landschütz	1·356	104	1·2	1·492	1·472–1·537
,,	1·360	144	1·5	1·505	1·488–1·528
,,	1·360	128	1·2	1·522	1·487–1·538
,,	1·360	126	1·2	1·519	1·492–1·550
,,	1·360	132	1·4	1·502	1·485–1·502
,,	1·360	200	1·8	1·519	1·510–1·548
Krebs	1·356	200	1·8	1·523	1·506–1·543
,,	1·356	80	1·8	1·423	1·416–1·443*
,,	1·358	200	3·2	1·451	1·447–1·459*
,,	1·358	140	1·6	1·490	1·475–1·509
,,	1·359	150	1·6	1·499	1·484–1·521
,,	1·358	150	1·8	1·484	1·471–1·500
,,	1·358	188	2·0	1·499	1·488–1·515
,,	1·358	180	1·8	1·508	1·494–1·527
Sarcoma 1	1·360	170	1·5	1·520	1·510–1·558
,,	1·361	120	1·2	1·536	1·489–1·585
Crocker	1·359	80	1·6	1·434	1·426–1·445*
,,	1·359	52	1·3	1·420	1·411–1·430*
EL4	1·361	84	0·8	1·510	1·488–1·571

* These droplets have refractive indices lower than any pure lipid substance, even allowing for maximum error in measurement.

of the 'agranular' double laminae known as Sjöstrand γ-membranes in the cytoplasm; which Chou and Meek (1958) proved conclusively were associated with the phospholipid droplets in the neurones of *H. aspersa*. Further study of the fine structure of the strains in which the phospholipid is particularly abundant (e.g. Krebs & Crocker) with the improved fixation techniques employed by these workers (involving the use of calcium to stabilize these membranes), would seem to be very desirable.

CONCLUSIONS

The investigation was carried out on ascites cells of a variety of different strains, of carcinomatous, sarcomatous, and leukaemic origin, and on solid tumours grown from most of the strains also representing these same three origins. The most noteworthy result is that the physical and chemical properties of the cells of none of these differ from one another in any important respect: nor is there any difference between the loose ascites cells and the cells of the solid tumours grown from them. The cells of leukaemic origin were different from the rest in their smaller size, their smaller lipid droplets and the absence, in almost all instances, of a recognizable idiozome region; but the physical and chemical properties of their components seemed to be the same as that of the remainder. It is therefore possible to obtain some sort of overall picture of a 'typical' mouse ascites cell which embraces the properties of nearly all the material investigated. This is illustrated in fig. 3.

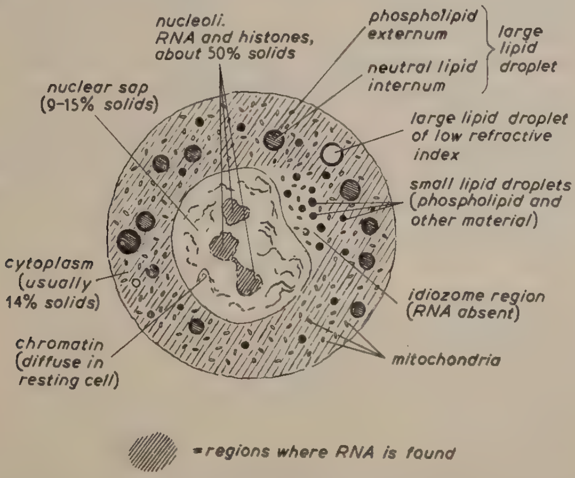
nucleoli. RNA and histones, about 50% solids
phospholipid externum
neutral lipid internum
large lipid droplet
nuclear sap (9–15% solids)
large lipid droplet of low refractive index
small lipid droplets (phospholipid and other material)
cytoplasm (usually 14% solids)
idiozome region (RNA absent)
chromatin (diffuse in resting cell)
mitochondria
▨ = regions where RNA is found

FIG. 3. A diagram illustrating the nature of the visible regions of a typical ascites tumour cell, based on refractometric studies of living cells and histochemical tests on sections of cells. Since the sizes of the individual cells vary in the different strains of ascites cells and also within a single population, the sizes of the inclusions relative to that of the whole cell are not necessarily exactly those depicted in this figure.

It is also probably justifiable to draw the conclusion that the physical and chemical properties of ascites cells, in general, differ very little from those of the solid tumours from which they are derived.

The demonstration of the presence of phospholipid in these ascites and solid tumour cells, and its localization within the large and small lipid droplets, appears to be a new finding.

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APPENDIX 1

Some particulars of the mouse asites tumours investigated

(From data supplied by Professor P. C. Koller, of the Chester Beatty Research Institute, Royal Cancer Hospital, London)

Designation	*Histological type*	*Mode, laboratory, and date of origin*	*Tissue and site of origin*	*Date and place of conversion into ascites*	*Host strain of mice*
Ehrlich	carcinoma	spontaneous, Frankfort, about 1900	subcutaneous	Stockholm, 1949	C57
Landschütz	carcinoma	spontaneous, Frankfort, about 1900	subcutaneous		C+
Krebs 2	carcinoma	spontaneous, Copenhagen, 1932	subcutaneous	Stockholm, 1950	C57
Sarcoma 1	sarcoma	induced, University of California, 1947	unknown	Stockholm, ? 1955	strain A (albino)
Crocker (S180)	sarcoma	spontaneous, Crocker Institute, Columbia University, about 1917	unknown	Chester Beatty Research Inst. London, 1957	non-specific
EL4	leukaemia	induced (X-rays), Guy's Hospital Medical School, London, 1945	lymph gland	Chester Beatty Research Inst. London, 1957	C57
C+Leukaemia	leukaemia	spontaneous, Chester Beatty Research Inst. London, 1957	spleen	Chester Beatty Research Inst. London, 1957	C57 C+

All early records of the history of the Ehrlich tumour were destroyed in the allied bombing in 1944.
The Landschütz tumour is now known to be a hyperdiploid variant of the parent Ehrlich strain (see Tjio and Levan, 1954).

APPENDIX 2

Summary of the histochemical and other tests performed on the sections of ascites cells, and the conclusions drawn from them

Staining and fixation	*Chromatin*	*Nucleoli*	*Cytoplasm, except in the idiozome region*	*Cytoplasm in the idiozome region*	*Small globules in the cytoplasm (mostly in the idiozome region)*	*Large globules in the cytoplasm*		*Mito-chondria*
						Externum	*Internum*	
Haematoxylin and eosin, Zenker 18 h	blue	blue	pink	pink	0	0	0	0
Feulgen, Zenker 18 h*	purple	0	0	0	0	0	0	0
Basic fuchsine, Zenker 18 h	+	+	+	0	0	0	0	0
Pyronine and methyl green, Zenker 3 h	blue-green	red	pink	0	0	0	0	0
Pyronine and methyl green, Zenker 3 h treated with rhibonuclease	blue-green	0†	0	0	0	0	0	0
Sakaguchi, Zenker 18 h	0	+	+	+	0	0	0	0
Metzner, Altmann and Helly	0	+	0	0	0	0	0	+
Sudan Black and IV, Formaldehyde-calcium	0	0	0	0	+	+	+	0
Acid haematein, Formaldehyde-calcium	+		0	0	+	+	0	0
Acid haematein with pyridine extraction, Formaldehyde-calcium	+	0	0	0	0	0	0	0
Cain's Nile blue, Formaldehyde-calcium	0	0	0	0	faint blue	blue	pinkish‡ blue	0
PAS, Formaldehyde-calcium	0	0	0	0	+	0	0	0
Neutral red (supravital)	0	0	0	0	+	+§	0	0
Conclusions	DNA	RNA and sometimes histones	RNA present	probably acidophil material	phospholipid and other material	phospholipid	neutral lipid	. .

* Feulgen control negative.

† Nucleoli occasionally pinkish.

‡ Pink internum often obscured by blue externum.

§ Externum not obviously stained in many large globules.

A Cytological and Histochemical Study of the Stria Vascularis of the Guinea-pig's Ear

By JOHN T. Y. CHOU

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With one plate (fig. 1)

SUMMARY

The cells of the stria vascularis of the guinea-pig have been studied morphologically and histochemically. The stria vascularis consists of three layers of cells with blood-capillaries. Apart from some yellowish pigments, the only cytoplasmic inclusions seen in the living cells and in the fixed materials are numerous rod-shaped mitochondria and spherical lipid globules. The mitochondria are about 1·5 μ in length with a diameter of 0·8 μ; they contain phospholipid. The lipid globules are about 1·0 μ to 1·5 μ in diameter; they contain phospholipid and a small amount of cerebroside.

The cytoplasm of these cells contains a small amount of diffused phospholipid and cerebroside.

INTRODUCTION

RECENT results of the analysis of the labyrinthine fluids suggest that the endolymph is formed and maintained by certain cells of the endolymphatic system of the inner ear. The present investigation is concerned with the histochemistry of the stria vascularis, which has been supposed to be involved in the formation of endolymph (Citron, Exley, & Hallpike, 1956).

The epithelial cells of the stria vascularis of the cochlea of the guinea-pig are attached to the spiral ligament as a ribbon (fig. 1, A) consisting of three layers of cells (fig. 1, B).

The extensive network of blood-capillaries in the stria vascularis and the position of the stria vascularis in the cochlear duct have been regarded as evidence of a secretory mechanism for supplying the endolymph. Shambaugh's histological study (1908) of the stria vascularis showed a true secretory, gland-like structure. Held (1926) confirmed this finding and expanded his view in support of Shambaugh's contention that the stria vascularis secretes the endolymph of the scala media. Von Fieandt and Saxén (1936) described the stria vascularis as epithelial in origin, and they considered that the endolymph is probably formed in the basal part of the cells. Saxén (1951), in his reinvestigation of the stria vascularis, stated that the columnar cells of the stria vascularis have broad, branched protoplasmic feet which are often closely connected with capillaries. From this feature and the well-developed 'Golgi net', he concluded that the cells of stria vascularis have a secretory function.

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In biochemical studies Smith, Lowry, and Wu (1954) showed that the utricular endolymph of the guinea-pig has a high potassium and low sodium content and it is in this respect comparable to intracellular fluid. Citron, Exley, and Hallpike (1956) confirmed their finding and suggested that the endolymph is probably produced by the secretory activity of specialized cells. Whether these are to be found in the stria vascularis or in the succus endolymphaticus or in both, these structures nevertheless remains unsolved (compare Naftalin & Harrison, 1958).

The present study gives an account of the cytological and histological characteristics of the cytoplasmic inclusions of the stria vascularis. It is based upon the results of the study of the living cells by phase-contrast microscopy and vital staining.

METHODS

A number of coloured guinea-pigs and a few white ones were used in this investigation. For the study of the living cells of the stria vascularis, 0·5 ml of 25% urethane per 100 g of body-weight of the guinea-pig was injected into the peritoneum to produce anaesthesia. The animal's head was firmly immobilized and the tympanic bulla opened by a posterior ventral approach to expose the cochlea. A ribbon of spiral ligament together with the stria vascularis was removed from the cochlea and transferred to 0·9% sodium chloride solution containing 0·1% of 10% anhydrous calcium chloride. In the coloured guinea-pig the stria vascularis could be clearly seen as a grey band through the bone of the cochlea. The spiral ligament was separated from the stria vascularis by teasing under a binocular. The stria vascularis was transferred to a slide with saline and examined by direct and phase-contrast microscopy.

For supra-vital staining, neutral red, Nile blue, brilliant cresyl blue, Janus green, and Janus black were used. The dye was dissolved at 0·5% in distilled water and then diluted with saline to 0·05%. The cells of stria vascularis were stained in the diluted dye for 5 to 10 min, flattened under a coverslip, and examined by direct microscopy.

For the study of fixed material, the cochleae were decalcified in Kristensen's buffered formic acid decalcifying solution (Lillie, 1954) for a week after fixation.

Baker's standard Sudan black method (1944, 1949, 1956*b*) and Sudan black after fixation in Lewitsky-saline solution were used for general cytological

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FIG. 1 (plate). Photomicrographs of vertical sections of the stria vascularis of the guinea-pig.

A, a basal coil of the cochlea, showing Reissner's membrane (*r*) separating the scale media (*sm*) from the scala vestibuli (*v*); the basilar membrane (*bm*) separates the scala media from the scala tympani (*t*). The cells of the stria vascularis (*s*) lie on the spiral ligament (*sl*.).

B, haematoxylin and eosin preparation to show the three layers of cells [basal (*ba*), middle (*mi*) and surface (*su*) layers], and two transversely-cut capillaries (*c*).

C, standard Sudan black method to show the distribution of the lipid globules (*l*).

D, Altmann-Metzner's method to show the short, rod-shaped mitochondria (*m*).

E, acid haematein method, to show the distribution of phospholipid in the mitochondria (*m*) and lipid globules (*l*).

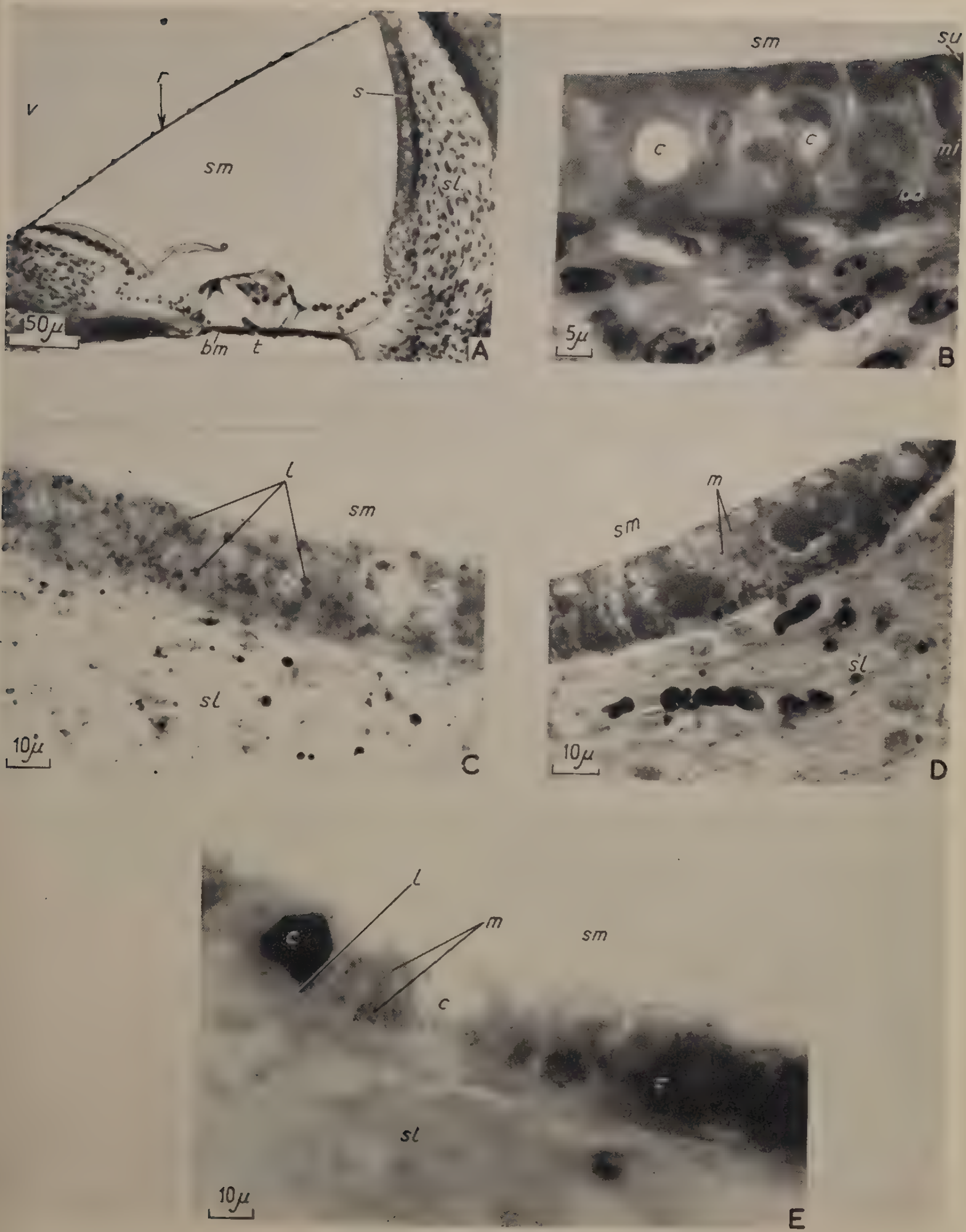

Fig. 1

J. T. Y. CHOU

study. Aoyama's (1929) and Weigl's techniques (1910) were also used in this investigation. Routine histological preparations (Masson's tricolor stain and Mallory's phosphotungstic acid / haematoxylin) were also made.

To show mitochondria, cochleae were fixed in Helly's, Altmann's, and Mann's fixatives. Sections were cut at 2 μ and were stained by Metzner's (1928) and Hirschler's (1927) methods.

The details of the histochemical tests are set out in a summary form in the Appendix (p. 81).

RESULTS

Unstained living cells

The stria vascularis consists of several layers of cells with a rich capillary system. By direct microscopy, small granules naturally devoid of colour are seen to be present in all the cells. These granules are mostly scattered near the periphery of the cell, but some are near the nucleus. They vary in diameter from 1·0 μ to 1·5 μ. The larger ones are slightly irregular in shape. In addition to granules there are light yellowish bodies in some of the cells. They are about 0·5 μ in diameter.

When examined by phase-contrast microscopy, the most striking feature of the cells of the stria vascularis is the presence of numerous short rods, which nearly fill the entire cytoplasm. These short rods are about 1·5 μ long and about 0·8 μ in diameter. The colourless granules appear darker than the cytoplasm and homogeneous; they are rather refractile. The pigmented bodies are also seen clearly.

Vitally stained cells

The granules are stained faintly reddish by neutral red, and blue by Nile blue and brilliant cresyl blue. Janus black and Janus green were also used. Occasionally the short rods were stained grey by Janus black, while the cytoplasm of the cells was not stained. These short rods are probably mitochondria.

Fixed preparations

In preparations stained with haematoxylin and eosin, Masson's tricolor stain, or Mallory's phosphotungstic acid haematoxylin, the stria vascularis appears to be stratified columnar epithelium (fig. 1, B). It consists of three layers of cells but the cell boundaries are extremely ill-defined.

Basal layer. Owing to the absence of a distinct basement membrane, the cells of the basal layer of the stria vascularis are not sharply separated from the underlying spiral ligament. They are about 16 μ to 18 μ long with elongated nuclei about 7 μ in length.

Middle layer. The cells of the middle layer are fusiform or columnar, each cell being about 16 μ to 20 μ long and having a large, oblong nucleus about 8 μ to 9 μ in length with 2 or 3 nucleoli. Abundant capillaries form loops in this layer.

Surface layer. The surface layer of the stria vascularis, that is the marginal cells which come in contact with the endolymph, are rectangular. They contain very few pigmented bodies. The cells are about 10 μ long, have oval or round nuclei about 4·5 μ in diameter, and are always situated near the surface of the cell. The surface of the stria vascularis is smooth.

Baker's standard Sudan black and Sudan black after Lewitsky-saline fixation (Baker, 1949) coloured the granules of all the cells of the stria vascularis blue-black (fig. 1, C), except the pigmented bodies. The cytoplasm is also slightly blue. When Weigl's technique was applied to these cells the cytoplasm became grey. Silver was deposited in the cytoplasm by the method of Aoyama without definite arrangement. No classical appearance of 'Golgi apparatus' was shown by either of these methods.

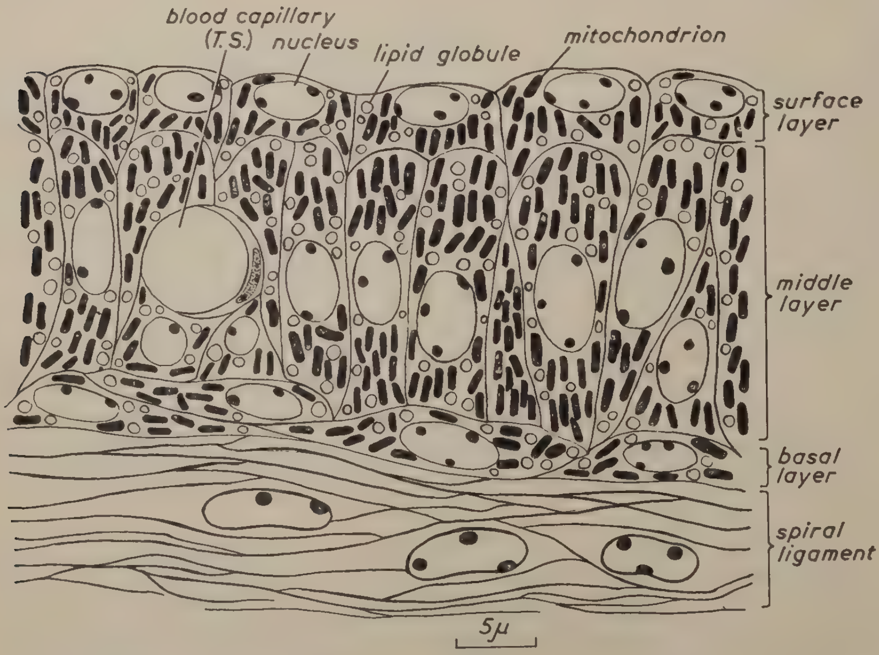

FIG. 2. Diagram to show the distribution of the mitochondria and lipid globules in the middle region of the stria vascularis of the guinea-pig.

The mitochondria of the cells of the stria vascularis are shown by Metzner's method and Hirschler's method as short rods. They are present in all the cells. In the middle region of the stria vascularis the mitochondria are mostly short rods, but a few of granular type are also present (fig. 1, D). The rods are arranged with their long axes parallel to the length of the cells. The mitochondria are mostly granular in the cells of the upper and lower regions of the stria vascularis. The number of the mitochondria in the stria vascularis is very high. An impression of the characteristic arrangement of the mitochondria in the middle region of the stria vascularis is shown in the form of a diagram (fig. 2).

Histochemistry of the cells of stria vascularis

Two inclusions formed the main subject at the histochemical investigation, namely, the granules and the mitochondria. A study of the pigmented bodies is not included in this paper.

The granules in the cells of the stria vascularis react positively to Sudan colouring agents (fig. 1, C), and this suggests that they consist of lipid. They give a positive reaction to Baker's acid haematein test for phospholipids, but negative after pyridine extraction. When a piece of stria vascularis is fixed in cold acetone and coloured with Sudan black, the lipid granules become pale grey; they cannot be coloured by Sudan black or any other Sudan colouring reagents if the tissue is extracted with hot acetone in a Soxhlet extraction apparatus. This fact suggests the presence of cerebroside. The pale colour indicates the presence of a very small amount of cerebroside. Cain's Nile blue test (1947) shows that the lipid in the granules is not neutral lipid. The lipid granules are negative to Windaus's and to Liebermann's tests and also to protein tests. The lipid seems to be evenly distributed in the granules. No crescents or ring appearances are seen.

By the use of Baker's acid haematein test it can be demonstrated that the mitochondria contain a great amount of phospholipid (fig. 1, E).

In general, the cytoplasm of the cells of stria vascularis gives a weakly positive reaction to the coupled tetrazonium test. Positive results were also given by Baker's Hg/nitrite test (1956*a*) and the Sakaguchi test (Baker, 1947). The PAS reaction is very weakly positive, and it is still so if the section is treated with saliva before the test. Since the cytoplasm of these cells is not chromotropic and is weakly positive to the Sudan colouring agents after cold acetone fixation, the PAS-positive reaction is perhaps due to a very small amount of diffused cerebroside. Since the cytoplasm is also weakly positive to the acid haematein test, it presumably contains diffused phospholipids.

The cytoplasm of the cells of stria vascularis is basiphil, colouring pink with pyronine in the pyronine / methyl green technique (Jordan & Baker, 1955). The basiphilia does not appear when the section is previously heated with salivary ribonuclease (Bradbury, 1956), and must therefore be presumed to be due to the presence of ribonucleic acid.

Usually two nucleoli are present in each nucleus. The chromatin of all the nuclei of stria vascularis reacts strongly to Feulgen's test.

Citron and Exley (1957) found that in contrast to the endolymph in fishes (Vilstrup & Jensen, 1954), that of guinea-pig has a low viscosity and low amino-sugar content. This finding is of interest in connexion with the apparent absence of both mucopolysaccharide and metachromasy in the stria vascularis.

DISCUSSION

The major finding of the present study is that the cytoplasmic inclusions of the stria vascularis of the guinea-pig consist of lipid granules and a large number of mitochondria; some yellowish bodies are also present in a few cells.

The lipid granules contain phospholipid and a small amount of cerebroside.

As would be expected the mitochondria contain phospholipid. The basiphilia of the cytoplasm of these cells is due to the presence of ribonucleic acid. Evidence was also obtained that the cells are rich in arginine and tyrosine.

Chromotropic granules, reported by Plotz and Perlman (1955) in the cells of stria vascularis of the bat, are not found in the epithelial cells of the stria vascularis of the guinea-pig.

Smith (1957) studied the cells of the stria vascularis of the guinea-pig with the electron microscope. She reported that small vesicles and short rod-shaped mitochondria were numerous in the cells of the stria vascularis. The present investigation has not only confirmed this, and has also provided some chemical information about the cytoplasmic inclusions of these cells. Smith also identified several groups of membranous in her electron micrographs as 'Golgi apparatus'. She gave no evidence of the presence of any 'Golgi apparatus' in the living cells. Chou and Meek (1958) showed that phospholipid globules in other tissues tended to break into crescentic membranous structures when buffered osmium tetroxide was used; calcium ions tend to preserve their spherical shape (compare Baker, 1958). It is possible that the objects which Smith referred to as 'Golgi apparatus' were either distorted lipid globules or the diffused phospholipid.

Saxén (1951) studied the cells of the stria vascularis and the epithelium of the prominentis spiralis of man and the dog. He considered the well-developed 'Golgi nets' in his preparations as evidence of a secretory function. These structures appeared in his photomicrographs as aggregations of spheres. He did not study the living tissue.

Nogi (1958) noticed the numerous mitochondria in the cells of the stria vascularis of the rabbit and also reported that there were structural changes of the mitochondria after electric stimulation of hypothalamus and intravenous injection of adrenalin or pilocarpine.

The present cytological and histochemical evidence, suggests that the epithelial cells of the stria vascularis are active cells and may well be related to the function of secretion or resorption of the endolymph.

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APPENDIX

A summary of the histochemistry of the stria vascularis of the guinea-pig

Test	Reference	*Results*		
		Lipid globules	*Mitochondria*	*Cytoplasm*
Standard Sudan black	Baker, 1944, 1956*b*	+++	—	+
Sudan IV	Herxheimer, 1901	+++	—	O
Windaus	Lison, 1953	O	—	O
Liebermann	Lison, 1953	O	—	O
Fischler	Pearse, 1954	+	—	O
Acid haematein	Baker, 1946	++	+++	+
Acid haematein control	Baker, 1946	O	O	O
Sudan black after hot acetone	Casselman & Baker, 1955	O	—	O
Sudan black after cold acetone	Casselman & Baker, 1955	+	—	+
Nile blue	Cain, 1947	+ (blue)	—	O
PAS	Pearse, 1954	O	O	+
PAS control	Pearse, 1954	O	O	O
PAS after saliva digestion	Pearse, 1954	O	O	+
Pyronin / methyl green	Jordan & Baker, 1955	O	O	+
Pyronin / methyl green control	Bradbury, 1956	O	O	O
Feulgen	Feulgen & Rossenbeck, 1924	O	O	O
Feulgen control	Feulgen & Rossenbeck, 1924	O	O	O
Coupled tetrazonium	Pearse, 1954	—	—	+++
Sakaguchi	Baker, 1947	—	—	+
Hg/nitrite	Baker, 1956*a*	—	—	+++
Alkaline phosphatase	Gomori, 1952	—	—	O
Acid phosphatase	Gomori, 1952	—	—	O
Basiphilia	—	—	—	++
Metachromasy		O	O	O
Acid mucopolysaccharide	Steedman, 1950	O	—	O

KEY. +++, strong reaction; ++, moderate reaction; +, weak reaction; O, negative; —, no observation.

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A Comparative Histochemical Study of the 'Golgi Apparatus'

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SUMMARY

The purpose of this investigation was to find out the responses of Golgi's net in the neurones of vertebrates to various dyes and histochemical reagents, and to compare these responses with those given by the 'dictyosomes' of the cells of invertebrates. Dictyosomes are regarded by many cytologists as the homologue of Golgi's net. The young oocyte of *Limnaea stagnalis* was chosen as a cell that possesses typical dictyosomes, which have recently been examined by histochemical methods.

The object that presents the characteristic Golgi pictures in the neurones of vertebrates is essentially different, not only in ultrastructure but also in its chemical composition, from those that represent in life the 'dictyosomes' of invertebrates. The dictyosomes of the oocyte react positively to tests for phospholipid and cerebroside, whereas Golgi's net is negative to these tests. Tests for arginine and for ribonucleic acid are positive for Golgi's net, but not for the dictyosomes of the oocyte. The dictyosomes are intensely stained by Rawitz's inversion staining technique and also by acid fuchsine (Metzner) and by iron haematoxylin, but these techniques do not show the net in the neurones of vertebrates. Golgi's net is resistant to embedding in paraffin after fixation in Clarke's (Carnoy's) fluid, but the dictyosomes are not. Neutral red is taken up during life by the object representing the dictyosomes, but not by the net of Golgi.

INTRODUCTION

IT is well known that when 'Golgi' impregnation methods (silver nitrate or osmium tetroxide) are applied to the neurones of vertebrates, a black network is seen; this is commonly called the 'Golgi apparatus' after its discoverer (Golgi, 1898). In many other kinds of cells the Golgi techniques do not produce a network; but instead small, separate rods and crescents, usually associated with a chromophobe substance, are seen. This is regarded by many zoologists as the typical form of the Golgi apparatus in most cells of invertebrates. These rods and crescents ('dictyosomes') are believed by many workers to represent, in a somewhat distorted form, the spherical or subspherical bodies that colour in life with neutral red (Parat, 1928; Young, 1953, 1956; Chou, 1957*a*; Baker, 1959; Malhotra & Meek, 1961). These neutral red bodies often show a differentiation into a surface layer of lipid nature and a non-lipid interior (Baker, 1959; Malhotra, 1960*b*). It is this lipid surface that selectively reduces osmium tetroxide or silver nitrate in Golgi methods. The blackening of the surface layer of the dictyosomes has led many zoologists to homologize these bodies with the network seen in the neurones of vertebrates, and indeed the word *dictyosome* (net-body) begs the question by asserting this homology. As a result, many cytologists consider that a homologue of Golgi's net occurs in all animal cells.

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It is the purpose of this paper to consider whether the 'dictyosomes' of the cells of invertebrates are in fact similar in their reactions to Golgi's net. It is thought best to select a particular cell that contains typical dictyosomes, and to compare their reactions to various dyes and histochemical reagents with those given by the object that is blackened to produce Golgi's net in the neurones of vertebrates. The cell selected for this comparison is the young oocyte of the pond-snail, *Limnaea stagnalis*. The structure and chemical composition of the 'dictyosomes' in this cell have recently been investigated in some detail (Malhotra, 1960*c*).

RESULTS

The neurones of the dorsal root ganglia of the mouse show a typical net (apparatus) of Golgi.

The object that other authors (Gatenby, 1919; Bretschneider & Raven, 1951; Raven, 1958) have called the Golgi apparatus ('dictyosomes') in the early oocyte of the pond-snail, *L. stagnalis*, consists of small, separate objects dispersed in the cytoplasm. In Golgi preparations they mostly appear in the form of rods and crescents, in association with chromophobe substance. Attention has been concentrated on early oocytes in which the yolk has not yet appeared.

If the tissue is fixed in Helly's (1903) fluid and postchromed, the dictyosomes of the oocyte can be intensely stained by acid fuchsine in Metzner's technique for mitochondria (1928), or by iron haematoxylin. These techniques, however, do not show the apparatus in the neurones of most vertebrates (Malhotra, 1959; Casselman & Baker, 1955). The dictyosomes are also strongly stained by basic fuchsine, used after mordanting in tannic acid and potassium antimonyl tartrate according to Rawitz's 'inversion staining' method (Przełęcka, 1959). The apparatus of the neurone is not generally shown by this method (Baker, 1959); it may occasionally be very feebly coloured.

The dictyosomes are coloured by Sudan black (Baker, 1949). They react positively to the acid haematein (AH) test, which becomes negative after extraction with pyridine (Baker, 1946); the results thus indicate the presence of phospholipid. Though one would expect the presence of lipid in an object that has a remarkable affinity for silver or osmium, the apparatus of the neurone is not coloured by Sudan black nor by the AH test (Casselman & Baker, 1955; Malhotra, 1959). Even when a powerful unmasking agent, such as cadmium chloride or mercuric chloride (Clayton, 1959), was used as a fixative in place of formaldehyde/calcium in the standard AH test, the apparatus did not show; it was coloured only very feebly with Sudan black. It is difficult to demonstrate lipid in this object by *in situ* histochemical methods.

The dictyosomes react positively to the periodic acid/Schiff (PAS) test of McManus (1948); the reaction is strong whether the test is applied to paraffin sections of tissue fixed in Zenker (1894) or gelatine sections of ovotestis fixed in formaldehyde/saline (Baker, 1949). The PAS reaction becomes weaker after digestion in saliva. The PAS test is also positive after extraction with cold or hot acetone (Casselman & Baker, 1955), though it becomes weaker when hot acetone is used. Sudan black also colours the dictyosomes more intensely after fixation in cold acetone than in hot. The apparatus of the neurone also responds positively to the PAS test, but the reaction is much weaker than in the dictyosomes of the oocyte. The reaction is also positive, though very weak, after saliva treatment. No positive reaction is seen in the apparatus of the neurone when the PAS test is applied to tissue fixed in acetone, hot or cold; Sudan black also does not colour the apparatus after this fixation.

The dictyosomes are blued by the Nile-blue test (Cain, 1947), while the apparatus of the neurone is not coloured at all.

The dictyosomes resist embedding in paraffin after fixation in Zenker; this suggests the presence of protein. However, when the oocyte is subjected to tests for amino-acids, namely, Sakaguchi (Baker, 1947) for arginine and Hg/nitrate (Baker, 1956) for tyrosine, a positive reaction is seen throughout the cytoplasm, but the dictyosomes cannot be differentiated. The apparatus of the neurone, on the contrary, is positive, though weakly, to the Sakaguchi test, but not to the Hg/nitrite test.

The apparatus of the neurone is intensely coloured by basic dyes (Malhotra, 1959, 1960*a*). The basiphil material can be extracted by ribonuclease (Bradbury, 1956) or trichloroacetic acid (Pearse, 1954). In contrast to this, the dictyosomes are not stained by basic dyes.

If Clarke's fluid (Clarke, 1851; Carnoy, 1886) is used as fixative and the ovotestis embedded in paraffin, the dictyosomes of the oocyte are not revealed in the sections. On the contrary, the apparatus of the neurone is resistant to such a treatment and can be demonstrated by colouring with basic dyes.

Neutral red is taken up by the dictyosomes of living cells from salt solutions. When this vital dye is used on the neurone of vertebrates, it appears in the small spherical bodies dispersed throughout the cytoplasm (Baker, 1944; Thomas, 1948; Malhotra, 1959), but the apparatus is not dyed.

Discussion

These results indicate that the objects commonly described as Golgi apparatus (dictyosomes) in the oocyte of the pond-snail are very different in their chemical composition from the structure that bears this name in the neurones of vertebrates. The objects representing in life the dictyosomes of the oocyte are similar in their form and chemical composition to the lipid globules of the neurones of vertebrates (Casselman & Baker, 1955). The lipid globules of these neurones are quite different from the structure that is blackened by Golgi methods to produce Golgi's net (Malhotra, 1959). In the neurones of invertebrates, however, the objects that produce the appearances of dictyosomes are the lipid globules that colour in life by neutral red (Parat, 1928; Young, 1953, 1956; Shafiq, 1954; Chou, 1957*a*; Chou & Meek, 1958; Malhotra & Meek, 1961). These are also histochemically similar to the dictyosomes of the oocyte of the pond-snail (Shafiq & Casselman, 1954;

Chou, 1957*b*; Malhotra, 1960*b*, though there may be slight differences in the chemical composition of the lipid globules in different cells.

The Golgi apparatus of the neurone of vertebrates differs from those of other cells not only in chemical composition, but also in its ultrastructure (Malhotra & Meek, 1960). Many electron microscopists consider that the Golgi apparatus of all kinds of cells is represented in micrographs by a system of closely packed, smooth-surfaced membranes, arranged in pairs (γ-cytomembranes of Sjöstrand, 1956), often seen in relation with vacuoles or vesicles (see Palay, 1958; Oberling, 1959, for references). Though elements of this system have been described in electron micrographs of the neurones of vertebrates (Palay & Palade, 1955; Hess, 1955; Malhotra & Meek, 1960), it remains to be proved that they form a reticulum corresponding to what is seen in this cell by routine Golgi techniques for light microscopy. This system is very sparse in electron micrographs and not nearly abundant enough to produce the massive net of Golgi (1898). Recently it has been suggested that this net is more likely to be formed by a deposit of silver on the membranes of the endoplasmic reticulum (Malhotra & Meek, 1960). The small ribonucleo protein particles (Palade & Siekevitz, 1956) present on and in between the membranous cisternae of the endoplasmic reticulum are presumably responsible for the basiphil nature of the Golgi apparatus in these neurones (Malhotra, 1959; David & others, 1960).

It would thus appear that the structures that are commonly called Golgi apparatus in the neurones of vertebrates and in the oocyte of *L. stagnalis* have nothing in common, except that they reduce osmium tetroxide or silver nitrate in arbitrarily devised techniques that are devoid of histochemical validity.

I am grateful to Dr. J. R. Baker, F.R.S., for the help and valuable advice given during the course of this work. Professor Sir A. C. Hardy, F.R.S., has been very kind in providing laboratory facilities.

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Phase Microscope Studies of Living Blood-cells of the Tunicates under Normal and Experimental Conditions, with a Description of a New Type of Motile Cell Appendage

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With one plate (fig. 4)

SUMMARY

The living blood-cells of tunicates have been studied by means of phase contrast microscopy. The species used were *Ascidia atra Lesueur*, *Ecteinascidia turbinata*, and *Clavelina picta*, all of which are common in the Bermuda region. Blood-cells in blood taken directly from the heart, or from the vessels, or elsewhere, particularly in the tunic of *Ecteinascidia turbinata* and *Clavelina picta*, were observed for periods ranging up to 24 h.

A new type of appendage is described for those cells which we have called vacuolate cells ('Q cells' of Fulton, 'signet-ring' and 'colorless morula cell' of George). Individual cells show from 1 to 5 appendages, which are beaded and actively undulate. Fragments from the appendages continue to undulate. Such fragments bear some resemblance to blood-platelets of higher vertebrates in their strongly adhesive properties.

In view of the morphological features, motility characteristics, and apparent functions, the terms *lymphocyte* and *macrophage* are considered appropriate for the finely granular and coarsely granular amoebocytes. No active swimming motions of the orange cells, nor of any other corpuscles were seen; but all of the types of cells, coloured and colourless, with the exception of the vacuolate cells, exhibit amoeboid progression.

No transformation of vacuolate into green cells was brought about in our experiments with varying strengths of acid, contrary to the earlier findings of Fulton. Many types of blood-cells in the tunicates have 'fixed cell' counterparts in the tissues. It is suggested that the large 'bladder cells' of the tunic of *A. atra Lesueur* may be such counterparts of the vacuolate cells of the blood.

INTRODUCTION

THE peculiar interest which attaches to the tunicates as primitive members of the group Chordata and the unusual biochemical and physiological features of their circulatory system (Henze, 1911, 1912, 1913; Hecht, 1918) have stimulated several studies of the blood of these organisms.

The presence of compounds of the element vanadium in the blood-corpuscles and the periodic reversal, at intervals of a few minutes, of the direction of circulation of the blood, are among the distinctive features. Two facts are of more direct importance in relation to the cellular composition of the blood: (1) the existence of pigment-carrying blood-cells, and (2) the apparent lack of distinction in these animals of blood-cells from cells of tissue fluid and wandering cells throughout the tissues, indicating the probability of a very free interchange between the circulatory channels and the tissues.

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The two authors who have contributed the most comprehensive articles on the blood-cells of tunicates have set up completely different classifications of the cell types, and their conclusions differ in regard to important characteristics of some of the types which obviously correspond in the two classifications. Such differences of opinion relate to the question of motility of the cell, presence or absence of a nucleus, functional significance, and genetic relationship to other types of blood-cells. The unresolved problems relating to the blood-cells seemed to us to lend themselves well to studies with the phase microscope, whereby details of structure and behaviour, invisible or very vaguely seen previously, might be expected to be brought out clearly.

Fulton (1920) concentrated his studies upon one species, the large black ascidian, *Ascidia atra*. He described 10 types of blood-cells or 'corpuscles'. Of these, three are pigmented: the green, orange, and blue cells. He divides the 7 types of unpigmented cells into two large groups, the motile or amoeboid cells and the non-motile or quiescent cells. In the motile group he designates three subdivisions A-1, A-2, and A-3 (A for Amoeboid). Under the quiescent group he designates four subdivisions Q-1, Q-2, Q-3, and Q-4 (Q for Quiescent). We shall discuss later some of the details of his description of these groups in comparison with our own observations.

Fulton gives approximate figures for the numbers of each type of cell, but emphasizes the difficulties involved in such counts. He found the green cells to constitute 60%; the Q cells (quiescent, colourless) 21%; the colourless amoebocytes, 16%; the blue cells, 2 to 3%; and the orange cells, 1 to 2%.

George (1926, 1930, 1939) studied the blood of *A. atra* also, but in addition carried out comparative studies within the group of tunicates, including work on *Perophora viridis*, *Ecteinascidia turbinata*, *Clavelina oblongata*, and *Symplegma viride*, all of which are colonial species.

For *A. atra* George (1939) lists 8 types of blood-cells. The pigmented cell types agree in general with those of Fulton. (The latter mentioned an infrequently seen 'brown cell', but did not list it in his summary. George failed to see such brown cells at any time.)

The unpigmented cells of George are: (1) the finely granular amoebocytes, which are said to resemble mammalian lymphocytes; (2) the coarsely granular amoebocytes, which have a peripheral layer of clear hyaloplasm and possess marked phagocytic properties; (3) the vesicular signet-ring type, a cell which has a single large vacuole and a thin peripheral layer of cytoplasm, thickened in the region where the nucleus is present; (4) the colourless morula cells, composed of 3 vacuolated lobes and a peripheral cap-like region with the nucleus and much of the cytoplasm; and (5) the amoeboid compartment cells, which show themselves to have a coarsely reticulated cytoplasm, at least after staining with neutral red, but which appear hyaline when alive and unstained.

George (1930) discusses the classification of Fulton and lists the correspondence of the categories in that classification to those in his own. For the unpigmented cells, George feels that the A-1 cells correspond to his own amoeboid compartment cells; the A-2 cells to his coarsely granular amoeboid cells. George does not seem to have a class corresponding to the A-3 cells, which Fulton describes as 'nearly spherical, highly granular and continually in rapid "swimming" motion'. The Q-1 and Q-2 cells of Fulton correspond to George's signet-ring type and the Q-3 and Q-4 cells (bilobed and trilobed respectively) correspond to George's 'colorless morula' type.

With these facts in mind, it will be less difficult to understand a comparison of our own observations with those of these authors and to consider the relative validity of the observations on these cell types which they made without the advantages of phase contrast microscopy.

MATERIALS AND METHODS

We have studied the blood-cells of three species of ascidians common in the waters around Bermuda: *A. atra Lesueur*, a large black solitary form, on which we have concentrated our attention; *E. turbinata*, a colonial type with zooids showing a bright orange pigment in the mantle and the gonads; and *C. picta*, a colonial form with zooids which are transparent except in the peripharyngeal zone and the endostyle, which are a fine purple. For the recognition and naming of these forms we have consulted Van Name (1902) and Berrill (1932).

Adult animals were used for the most part in this study. Twenty-four specimens of *A. atra*, ranging from 5 to 9 cm in length, were used, and a large number of zooids of *E. turbinata* and *C. picta*, each zooid from a different colony, were studied.

In the case of *E. turbinata*, where we found tadpoles readily available from several colonies, we made a survey of the blood-cells of the tadpole and of a few recently metamorphosed animals, for comparison with those of the mature forms.

The blood was obtained in either of two ways: (1) by cutting a flap in the tunic to expose the body of the animal, inserting an 0·8-mm oxalated capillary tube directly into the beating heart, and withdrawing a drop of blood; or (2) piercing the tunic and mantle with the detachable blade of a scalpel, never previously used, and picking up with a pipette a small portion of the cloud of blood which immediately poured forth into the sea-water. In the first method there was, of course, no dilution; in the second the dilution was with the sea-water into which the blood issued. Actually, the appearance and behaviour of the cells of the blood, as obtained by the two methods, are very similar. The blood removed by either method was placed directly on a No. 1, 22 × 50-mm cover-glass and a 22-mm square cover-glass was put over it. The cover-glasses with the blood were transferred to the mechanical stage of the microscope. The instrument used was the Zeiss inverted plankton microscope with phase-contrast condenser III Z/6. Bright field (positive) phase contrast was used.

Magnifications of × 400 and × 1000 were found to be most useful. In every case the preparations were examined over a period of many hours and in a number of instances in which the dilution method was used, activity of the cells continued for as much as 24 h.

We were interested in the possibility of observing the various types of blood-cells in some other tissue environment and made various attempts to do so with freshly removed tissues, including branchiae, body-wall, gonads, and tunic. It is in the tunic that by far the most successful observations of this type can be made. The ground-substance of the tunic of *C. picta* is almost completely transparent and the cells in it can be seen with the greatest clarity under phase. The tunic of *E. turbinata* has a considerable degree of transparency although the orange pigment somewhat obscures the picture. The tunic of *A. atra*, with its thick black appearance in the gross aspect, at first seemed somewhat discouraging; but thin hand-cut sections revealed a great concentration of pigment peripherally and a transparent quality of all of the deeper layers, with the cells readily observable.

In addition, pieces of all of the tissues studied in the living animal were fixed in 10% formalin and carried through the paraffin method. Sections were cut at 6 μ, and stained with haematoxylin and eosin or Malory's trichrome connective-tissue stain.

OBSERVATIONS

The blood of *A. atra* shows a constant preponderance of green cells, moderate numbers of amoebocytes and 'vacuolate' cells, and scattered blue cells and orange cells. We have not made an attempt to determine the exact numbers of the types in this study, but we would feel in general agreement with Fulton (1920), whose figures we have given above, although we must differ with him on the characteristics and probable relationship of the types.

With the phase microscope the amoeboid cells, both the so-called finely granular and coarsely granular types, show a tendency to adhere to the glass and to begin progressive motion almost as soon as the preparation is made. The changes in shape are rapid (fig. 1). A conspicuous feature is the presence on many of the cells of long, needle-like pseudopodia. These usually appear to be free of the glass and occasionally present a 'to and fro' motion which seems active rather than passive. A second conspicuous feature is a sudden production of clear, bleb-like pseudopodia from any portion of the surface of the cell, indicating progression in the direction toward which they protrude. The suddenness of formation of these blebs is a constant feature. The endoplasm then flows slowly into them.

There is a question in our mind as to whether some of the amoeboid cells may actually have filamentous projections as they float in the blood. At least, we have been unable to begin our observation rapidly enough after extravasation to see such cells only in a rounded condition. In this regard, the rather sluggish rate of flow and the periodic reversal of its direction might be of significance in not maintaining a rounded form.

The change of shape of the amoebocytes and their progressive motion persists for from one to several hours in our preparations and they show no tendency to disintegrate. In three instances we have seen division of amoebocytes. A constriction first appears and is drawn out into a long bridge. During this process, as seems general in dividing cells, the pseudopodia are drawn in and the two cells present a rather smooth outline until division is completed. As far as we could tell, these three divisions, seen in separate preparations from different specimens of the black tunicate, were normal and each gave rise to two amoebocytes of approximately equal size.

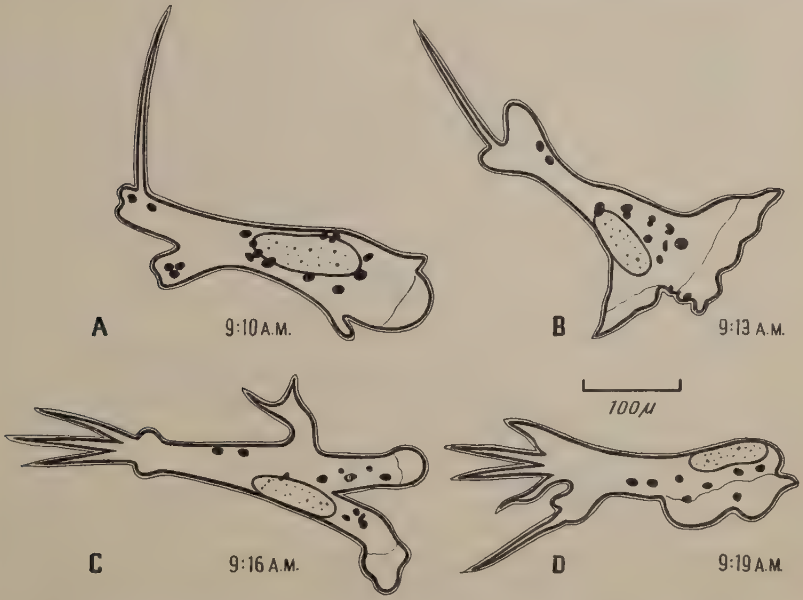

FIG. 1. A blue cell from the blood of *A. atra*, showing the rapid changes in form during progressive motion in a cover-glass preparation. Positive phase contrast.

The large amoebocytes vary somewhat in appearance. In many of them the cytoplasm appears as an almost homogeneous grey mass with only a greater clarity marking the blunt pseudopodia at their formation. In some, on the other hand, the ectoplasmic portion is sharply distinguished from the endoplasm, appearing as wide, clear sheets surrounding it. In such cases the endoplasm contains granules and particularly lipid droplets. That these two appearances are only two aspects of the same type of cell seems evident from the presence of intermediate forms, from the behaviour of the cells, and from the fact that the cells of the two appearances vary inversely in number in different specimens, the cells of the homogeneous type being more numerous when the granular is less numerous, and conversely.

The small amoebocytes, on the contrary, vary little in appearance from cell to cell or from animal to animal. They resemble the large lymphocyte of vertebrates in showing a clear nucleus, usually with a distinct nucleolus, and a fairly large amount of cytoplasm. The cytoplasm appears a homogeneous grey. Amoeboid activity occurs but seems far less marked than in the large amoebocytes.

Phase-contrast microscopy clearly reveals a third type of amoebocyte present in the blood. This is a cell with a strikingly reticulated cytoplasm (fig. 2, A). Dark bands form a very regular type of network, with a light grey, often almost entirely clear, cytoplasm in its interstices. The nucleus of such cells is usually seen without difficulty. It is rather small, contains powdery chromatin, and generally does not show a nucleolus. This type of amoeboid cell moves slowly and its ectoplasmic border is narrow and inconspicuous.

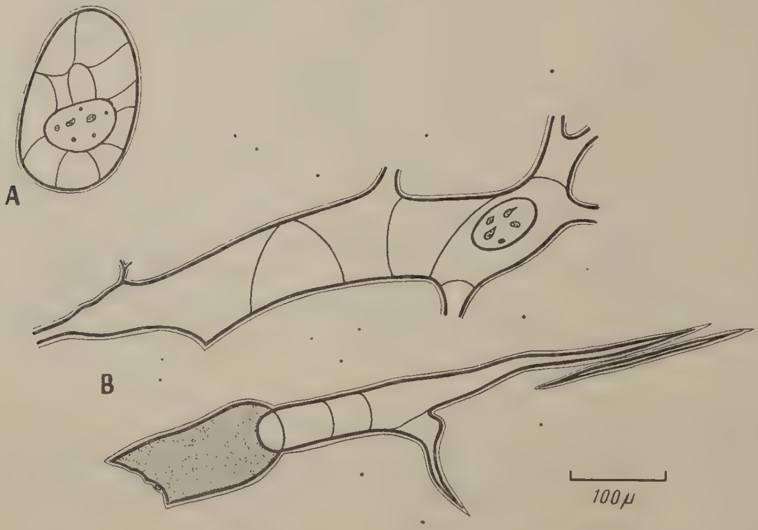

FIG. 2. Colourless compartment cells from *E. turbinata*. A, cell from the blood; B, cells from connective tissue of the tail of a newly hatched larva. Positive phase contrast.

The coloured cells of *A. atra*, green, blue, and orange, have been grouped together by earlier authors apparently simply because of the possession of colour. Actually, there seems to be a greater resemblance in morphological and behavioural features between some of the coloured types and the colourless types than between the different coloured forms. In this regard, the blue cell of the blood seems to us to be very similar to the large amoebocyte. In fact, apart from the presence of the blue pigment, its resemblance in form and motion to the homogeneous phase of the large amoebocyte is striking (see fig 1). The blue pigment itself is marked by a considerable variation in appearance, being present in some cells as minute granules of rather uniform size, in others as coarse spheroidal 'clumps' of material varying considerably in size. We shall describe later the appearance of these cells in the tunic and their probable relationship with those of the blood.

Before describing the other two types of coloured cells, we shall give our own findings on the group of cells which Fulton describes as Quiescent (Q-1, Q-2, Q-3, and Q-4) and which George apparently lists under at least two groupings, namely, the vesicular signet-ring cells and the colourless morula cells.

These cells are marked particularly by the presence of from one to three spheroidal vacuoles, which we find to have a light yellowish tinge, although the earlier authors appear to us justified in not including such cells as coloured cells on the basis of this faint colour. The vacuole apparently contains a highly refractive material which contrasts strongly with the light grey cytoplasm. The cytoplasm is always scanty but in some cases, particularly in the cells with one vacuole, it forms a well-defined cap, in which the nucleus is perceived by phase contrast with some difficulty.

The cells with one vacuole, which we shall call the *unilocular vacuolated cells*, are far more numerous, in a ratio of more than 10 to one, than the other two types of vacuolated cells combined (those with two vacuoles or *bilocular vacuolate cells* and those with three vacuoles or *trilocular vacuolate cells*).

A striking feature of the unilocular vacuolate cells is the presence in the vacuole of a solid body, a small spheroidal concretion which is constantly moving slowly from place to place within the vacuole, thus changing its position with relation to the cytoplasmic cap and the nucleus (fig. 3, A, C). In addition, there may be one or a few very minute granules which show Brownian motion. The consistency of the material in the vacuole would thus appear to be that of a non-viscous fluid.

Are the unilocular vacuolate cells actually quiescent? We have not seen any progressive movement by these cells, either in the blood or tunic. However, we have on a number of occasions seen lobe-like pseudopods formed from the crescent of cytoplasm, indicating at least an ability to change shape to a slight extent. The smooth spheroidal contour of the vacuole, however, has never been seen to change, a point to remember in relation to the contents of the green cells.

The completely new feature of the unilocular vacuolate cells which phase-contrast microscopy has revealed to us is the property which they have of putting out one or several appendages. There are long, thin, beaded processes which undergo a curious active undulation that may last for many hours. We first observed this phenomenon in a group of such cells in one of our early cover-glass preparations which had remained under the microscope for about 85 min.

We have since seen these appendages on vacuolate cells in preparations immediately after the blood had been removed. In fact, such preparations, when there is still a slight flow of fluid, seem to offer the finest view of them, and every individual cell may be seen to have from one to four such appendages extending out from the cell body. The length of the appendages varies, from about the diameter of the cell (7 to 9μ) up to 4 or 5 times this magnitude, and in exceptional cases even much longer. We have seen one appendage over 90 μ in length.

Observation of individual cells has permitted us to see these beaded appendages as they first appear, as short structures showing only two or three 'beads' and as they increase in length. We have not been able to ascertain fully, however, the exact manner of formation of the beads, whether new ones are formed at the base or at the tip of the appendage, although we incline toward the former location. Indeed, the terminal bead often is considerably larger than the others in the series.

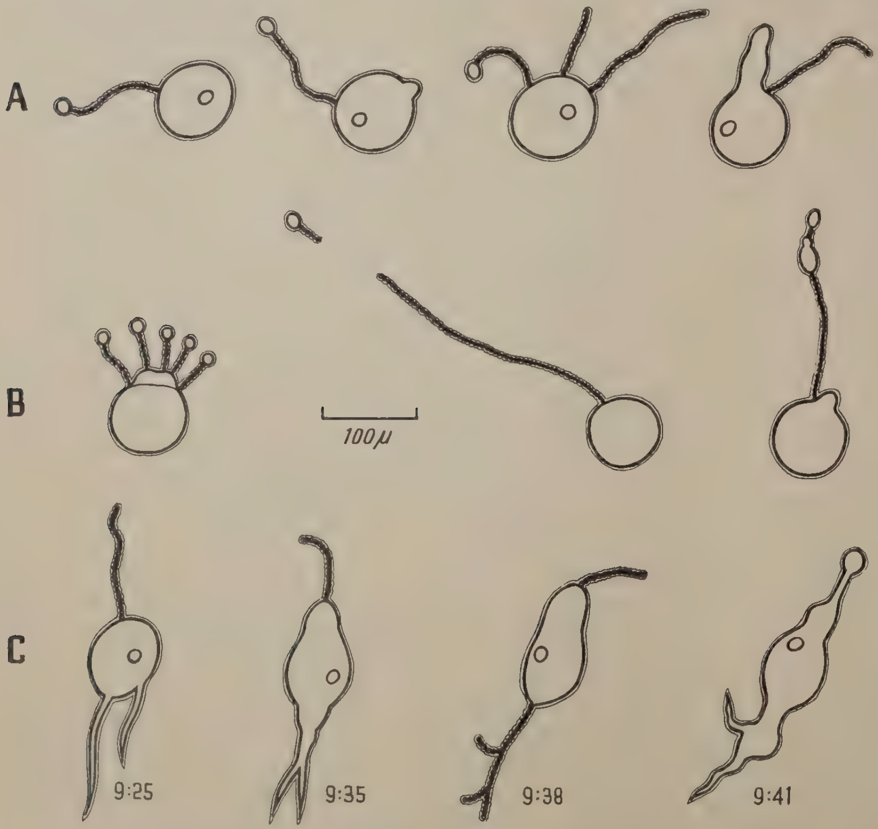

FIG. 3. Various aspects of the vacuolate cells of *A. atra.* A and B, variations in number and length of the motile processes. The central cell in B shows fragmentation of a process, the detached piece remaining active. C, changes in form of the body of a vacuolate cell occurring over a short period of time. Positive phase contrast.

During prolonged observations on individual appendages, we have seen many instances of an interesting phenomenon. This is the breaking off of several segments at the end of the appendage. Such isolated pieces actually continue the distinctive, actively undulating motion in almost the same manner as when attached to the cell. We have seen such isolated pieces many times and have observed them continuously over various periods. One piece of 5 segments became attached by its 'basal' segment to the glass and continued 'writhing' for 65 min.

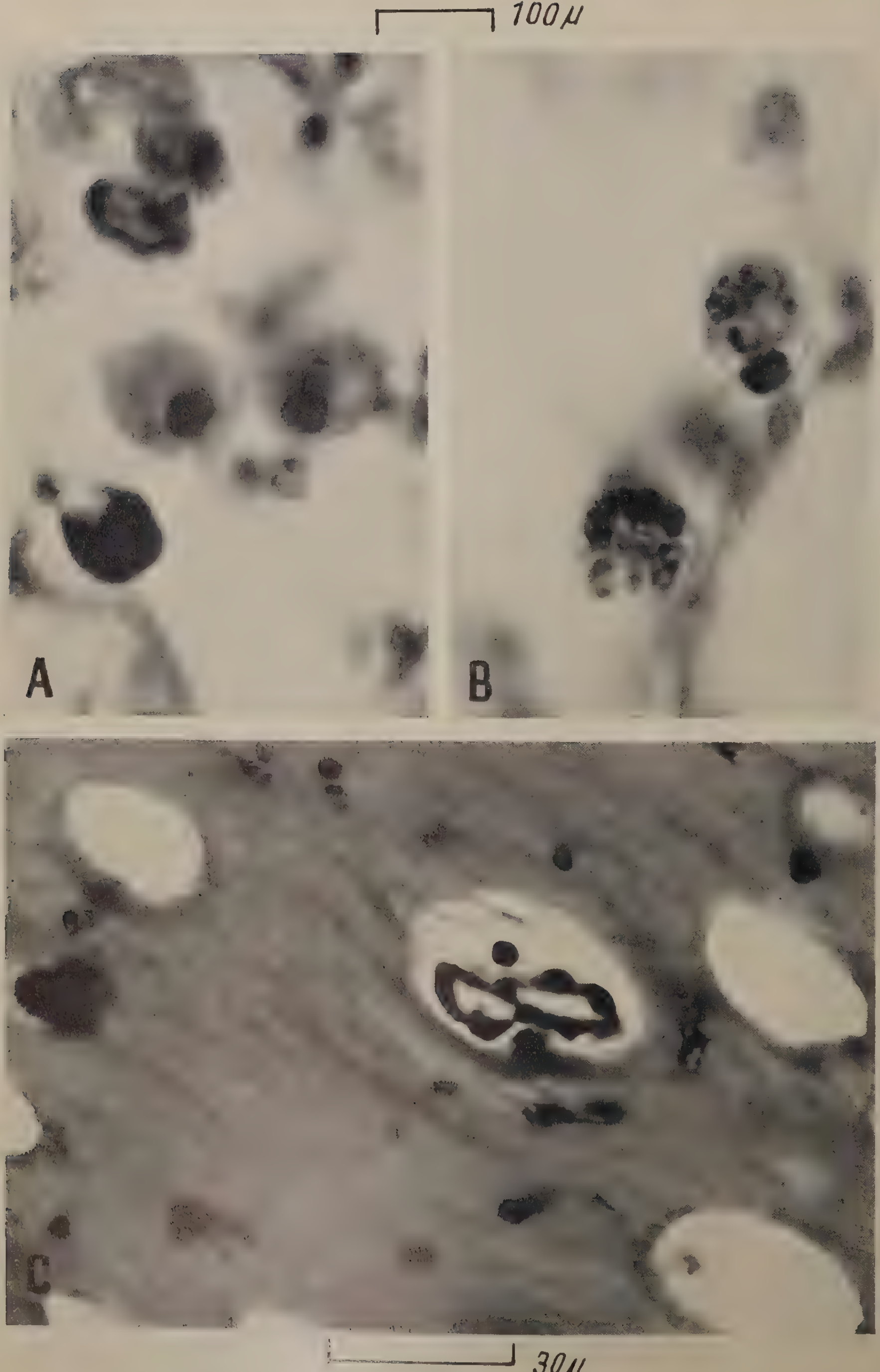

Fig. 4

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The beaded appendages appear to be formed from the cytoplasm of the unilocular vacuolate cells without any participation of the material of the vacuole, which remains undiminished in size. By positive phase-contrast microscopy the 'beads' appear black, the very short intervening portions of the appendage grey. We have never seen any of the yellow, refractive appearance of the vacuole in any of the appendages.

It is easily understandable how earlier workers, using direct microscopy, would not have seen the beaded appendages. We have obtained fields in which a number of such appendages could be seen with the greatest clarity by positive phase contrast, changed back then to the ordinary optical system, and found them at first to be entirely invisible. Knowing exactly where an appendage is, it can with difficulty be identified without phase contrast, but surely would be passed over readily.

The other two kinds of vacuolate cells, the bilocular and trilocular ones, resemble the unilocular in the appearance of the vacuolar material. We have only occasionally found in one of the locules a granule or concretion like that which is almost constantly present in the unilocular vacuolate cell, and we have not seen formation of appendages from them, although, since these cells are far less common, such formation may have been missed by us. In any event, however, if the appendages do occur on these other vacuolate cells, they would appear to be less common than on the unilocular ones.

The two remaining types of blood-cells are the green cells and the orange cells. The green cells, the most abundant of all of the types, are of about the same size as the unilocular vacuolate cells. Their most prominent feature, with phase contrast or without, is the presence in each cell of a number of somewhat wedge-shaped green bodies (fig. 4, B). These bodies are often irregularly placed but may present a sort of blastula-like arrangement, with the narrower portion of each wedge directed toward a clear central area.

We have not seen any concretions in the green cells nor any granules exhibiting Brownian motion. In fact, the green material of these cells appears to be a viscid substance, in contrast to the material in the vacuoles of the vacuolate cells.

Fulton described a transformation of his Q-2 cells into green cells, as a result of treatment of the cells with weak acid. We studied the effects of such treatment, using phase contrast and three different concentrations of acetic acid. A drop of acetic acid, 0·025, 0·5, or 0·1 N, was added to a drop of ascidian blood and the results studied. Preparations were made both with and without

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FIG. 4. (plate). Tissue of *A. atra*, fixed in formalin, stained with Harris's haematoxylin and eosin.

A, interior of a branchial vessel. Two large macrophages are seen near the centre of the field and a blue cell in the lower left portion.

B, interior of a branchial vessel. Two green cells are present, with a smaller macrophage above them. The green cell nearest to the centre of the field shows an eccentrically located nucleus.

C, tunic, showing a small blood-vessel, the lacunae of several of the large vacuolate cells, and the fibrillar appearance of the intercellular material.

a cover-glass. The only clear-cut change which we found in the vacuolate cells was with 0·1 N acetic acid. Here, there appeared to be a solution of the vacuolar contents, and there was rapid swelling and a pushing of the concretion to the periphery of the cell. We were chiefly interested in the effect in the vacuolate cells and we followed 100 of these cells for periods ranging from 5 min. to a half-an-hour, after use of each of the three strengths of acid. We did also observe the other cell types during such treatment.

Table 1 shows the changes in different types of cells at the various strengths of the acid.

TABLE 1

Effect on blood cells of A. atra *of treatment with acetic acid of different strengths*

Normality of acid	*Cellular Alterations*				
	Amoebocytes	*Green*	*Blue*	*Orange*	*Vacuolate*
0·025	Cessation of motion	No visible change	Cessation of motion	No visible change	No visible change
0·05	Tendency to withdraw Pseudopods	No visible change	Tendency to withdraw pseudopods	No visible change	Moderate swelling
0·1	Rounding up	Accentuation of outlines of green bodies within cell	Rounding up	Accentuation of outlines of orange bodies within cell	Marked swelling. Solution of vacuolate contents. Cessation of brownian motion. Concretion to periphery

The orange cells resemble the green cells in size and form. The contents of the cell, however, may be in the form of 'clumps' of orange pigment or in that of more separate granules and fusiform bodies. The clumps are more reminiscent of the bodies within the green cell but are not as definitely wedge-shaped and the 'blastula-like arrangement' has not been seen. The problem of motility of these cells will be discussed later.

A comparative study of the blood of *E. turbinata* and of *C. picta* shows great similarities to that of *A. atra* but also some important differences. Both of these species show the three types of amoebocytes, the three kinds of vacuolate cells, and the green cells. They both lack blue cells. *E. turbinata* also has conspicuous orange cells in the blood, lacking in *C. picta*. *E. turbinata* also has another type of cell, an amoeboid cell with its cytoplasm filled with refractive, colourless spheroids which roll over one another as the animal moves. *C. picta* has a brown cell in the blood, not seen in either of the other species. This cell has one to three vacuoles containing a brown fluid with granules presenting Brownian motion.

The green cells of these two colonial species resemble morphologically those of *A. atra* but the green substance itself is far paler and sometimes appears almost clear. The vacuolate cells of these two species also differ somewhat from those of the black tunicate. The crescent of the unilocular vacuolate cells is less prominent and the concretion in the vacuole is a less constant feature.

'Blood-cells' in other tissues of tunicates

We have confined our study of the blood-cells, or perhaps it would be better to say 'the blood-cell types', to tissues in which these may be distinguished in the living state. A concentration of orange cells on the branchial papillae of *A. atra* was seen in all the specimens which we examined. Such cells were very seldom identified, except within blood-channels, in the tunic of this same animal, while in *E. turbinata* the tunic shows numerous large orange cells which constitute the 'pigment cells' lending colour to the zooids. The orange 'cells' here are several times the size of those in the blood, have large numbers of fusiform orange granules, and send processes out widely into the surrounding tissue. While we were able to identify cell types in other tissues, we have been able to observe cell behaviour only in the tunic.

Cell motion seems to be at a minimum in the thick tunic of *A. atra* but to be very active in the tunic of both *E. turbinata* and *C. picta*. The preparations of the tunic of *A. atra* were made by cutting thin sections through its thickness and placing these on a cover-glass in a drop of sea-water. With the inverted microscope it was not necessary to place a cover-glass over the preparation.

Studied in this way with low power, the main mass of the tunic, all except a thin layer at the periphery, shows an almost transparent mass with the blue cells and greyish, fibroblast-like cells, scattered through it. Examined under higher powers, the transparent mass resolves itself into a mass of very large spheroidal cells which appear rather closely packed together. The substance of these cells is of an extreme clarity. In many of them a narrow crescent of light grey appearance can be seen. In nearly every one of them a concretion can be seen, strongly reminiscent of those in the unilocular vacuolate cells of the blood. The only difference is a greater tendency to some elongation or irregularity of the concretion in the cells of the tunic.

Closer study shows that a few of the tunic cells actually are bilocular or trilocular in character, adding further to their resemblance to the vacuolate cells of the blood.

The blue cells of the tunic resemble those of the blood in general appearance and in range of variation in the aspect of their granules. They seem, however, to be 'fixed cells'. We have been unable to see any motion whatever in them. Indeed, no motion by any cells outside the blood-channels has been seen in the tunic of this ascidian.

In the colonial species the thin, transparent tunic, just removed and placed in sea-water on a cover-glass, is a scene of great activity. All the types of blood-cells which are seen to move in preparations of the blood are seen here also and all are in motion. The green cells in the tunic are more active than those in preparations of the blood and indeed are fully as active as the large amoebocytes. Their activity helps to demonstrate the viscid character of the green substance, which flows with the cytoplasm, slowly separates into droplets by constrictions of the cell-body, and often shows slow re-fusion of such droplets, as of those of an oil of a thick consistency.

In the tunic of the two colonial species we have seen none of the large, clear, spheroidal cells so conspicuous in that of *A. atra*. The matrix is almost entirely clear, but a few delicate fibres are seen scattered through it in irregular fashion.

Blood of tadpoles of Ecteinascidia turbinata

In the tadpoles of *E. turbinata* the blood-vascular system and circulating blood are seen in the living animal. Free blood was readily obtained by separation of the tail from the trunk under the binocular dissecting microscope. Study of cover-glass preparations reveals the presence of small amoebocytes (lymphocytes), amoeboid compartment (reticulated) cells, green cells, and orange cells. These types of cells show the same kinds of motion as in blood from the adult specimens. Large amoebocytes and vacuolar cells appear to be absent.

A point of some interest was the finding in the clear tail-fins of the tadpoles of cells which have cytoplasm almost identical in appearance with the amoeboid compartment cells of the blood but which are fixed cells, as determined by prolonged observation. These cells also show a definite orientation in relation to the delicate fibres near to them, such that processes of the cells run parallel and in close relation to such fibres.

Discussion

The variation in the appearance of the large amoebocytes is noteworthy. We have described these cells as having in many cases a rather homogeneous cytoplasm, in others showing granules, lipid droplets, and even phagocytized cells. We have also pointed out that the cells with more inclusions generally show broad sheets of clear ectoplasm. While the differences probably are of a functional nature, it seems important to stress their occurrence. We are inclined to agree with George's change from the expression used in his 1930 paper, 'coarsely granular amoebocytes', to that used in his 1939 paper, 'macrophages'; for these cells surely are reminiscent, in structure and apparent function, of the macrophages of vertebrates, which they resemble also in the variety of aspect which they present.

Such large amoebocytes, with their differences in functional aspect, evidently are represented in Fulton's classification by the A-1 and A-2 cells, which he indicates as being of the same general size (10 to 15 μ in diameter in the living state) and as differing chiefly in the granularity of the cytoplasm of the A-2 cells.

A feature of the amoeboid motion of the large amoebocytes which has seemed prominent to us is the frequency of occurrence of a sharply pointed pseudopod oriented in the direction of travel. This is so definite in these cells that while progressing they may almost be said to show an 'anterior' and a 'posterior' extremity. Not infrequently the pointed end will develop a flexure a few μ back from its tip, and as it turns the remainder of the body will follow. Thus the direction of the point will indicate the direction in which the cell is progressing or toward which it is turning. A cell may go for considerable distances without changing its shape greatly. On the other hand, it may rest rather quietly in one area and repeatedly put out blunt, bleb-like pseudopods, undergoing an almost constant transformation of shape and appearance.

The small amoebocyte, or finely granular amoebocyte of George's earlier work, later called by him the lymphocyte (George, 1939) seems to correspond in Fulton's classification to the A-3 cell, which is 8 to 9 μ in diameter in the living state.

These cells resemble the lymphocytes of vertebrates not only in appearance but in manner of progression. During amoeboid motion they elongate but have a blunter 'anterior' end. The nucleus, as with the mammalian lymphocyte, is 'dragged' in the posterior end.

Our own third type of amoebocyte, the coarsely reticulated type, seems to have no parallel in Fulton's classification. We cannot doubt, however, that it does correspond to George's 'amoeboid compartment cell' (George, 1930, figs. 9, 10, 53, 63). This is in spite of the fact that to George these cells were 'hyaline when alive and unstained'. He used weak neutral red in sea-water, which brought out the reticulated cytoplasm and angular vacuoles after about half-an-hour. By phase contrast these features, ordinarily invisible, are seen very readily. Such cells possess both blunt and pointed pseudopods, formed from an outer layer of non-reticulated cytoplasm (ectoplasm).

Perhaps our most interesting finding in relation to amoeboid compartment cells is the discovery of their counterpart as 'fixed tissue' cells in at least one location, namely, in the tail-fin of tadpoles of *E. turbinata*, where we have described them (see above) as in intimate relationship with the fibres.

The relation between cells of the blood and fixed cells in the tissues becomes of much interest in regard to the blue cells and orange cells. In *A. atra* it is the blue cells that give the bluish-black colour to the tunic. Fulton (1920, p. 409) says: 'As far as can be ascertained there is no essential difference morphologically between the blue cells in the blood and the pigment cells in the test.' He does state also, however, that the pseudopodia of the test cells are never seen to contract. This we have confirmed in prolonged observations on thin pieces of test. To our mind such a difference is rather an important one, in that we seem to be dealing, in the test, with a fixed type of cell, which, therefore, is not the same as the blood-cell but rather its fixed counterpart.

The group of cell types which we have described as a series, namely the unilocular, bilocular, and trilocular vacuolate cells were treated in two quite different ways by Fulton and by George. Fulton, as we have seen, did consider them as a series, which he designated as the Quiescent Cells (Q-1, Q-2, Q-3, and Q-4). He recognized the large vacuole of the unilocular type (his Q-1 and Q-2) and the frequent presence of a small concretion within the vacuole. This concretion he called the 'excretory granule' and claimed to have seen it in process of elimination from the cell on several occasions. He pictures the 'granule' as a somewhat rectangular structure, while we have found it almost always to be spheroidal. We have watched some of these cells for several hours but have not been able to observe 'excretion' of the granule. Certainly the granule moves about rather freely in the liquid contents of the vacuole.

The bilocular and trilocular vacuolate elements were recognized by Fulton, and also later by George, to be individual cells, but George does not relate them to the unilocular form. Rather, he describes the unilocular vacuolate cells as 'signet-ring cells', has no special category for bilocular cells, and describes as 'colorless morula' cells the cells which we have spoken of as 'trilocular vacuolate'. While we do not feel that Fulton's system of naming these cell types by letters and numbers is a good one, we also feel that the terms 'signet-ring' and 'colorless morula' are not adequate descriptions of these cell types. The latter term, indeed, is misleading, since, although without that intent, it certainly would seem to indicate a group of several cells.

The striking feature of the unilocular cells, the active beaded appendages seen by phase contrast, were not seen by the earlier workers. What their significance may be, it is difficult even to speculate. It seems not unlikely to us, however, that they might have some 'usefulness' or function even in the circulating blood. The cells themselves possibly might require a stabilization mechanism, particularly as they do show a definite polarity. It is even tempting to speculate here on a possibly equilibratory function of the concretion. In any event, we do not think it likely that these appendages represent simply an 'abnormal' phenomenon in extravasated blood.

The apparent 'vitality' of these appendages is rather surprising, for we have found an occasional one in motion over 24 h after extravasation. We have seen such appendages undergo a separation of the terminal segments, a small chain of cytoplasmic beads thus being freed into the water. Such a chain *continues* its active undulating motion. In one case we continued to observe a chain of 5 'beads' for 65 min after its separation from the long appendage of a cell. In this case, and in a number of others, the proximal 'bead' of the isolated chain was seen to be attached to the glass, while the rest of its length continued to undulate.

One of the characteristics of the beaded processes suggests a possible function for them. This is the property of *adhesiveness* of the individual beads, which causes them to stick to the substrate. Such adhesiveness reminds us of the same characteristic as is found in the blood-platelets of higher vertebrates. The 'beads' resemble the platelets also in being fragments of cytoplasm derived from cells but continuing a certain independent existence after separation from the parent cell. Whether the role of these cytoplasmic fragments in tunicates may be related to clotting of blood, as in vertebrates, is a question which will require experimental studies for its elucidation.

Our study of the histological structure of the fresh tunic has indicated a rather striking resemblance between the vacuolate cells of the blood and the very large, so-called 'bladder-cells' of the tunic. The chordoid type of cell is found in the tunic of many ascidians (Seeliger & Hartmeyer, 1911), but of the three studied here only *A. atra* possesses such cells. These 'bladder-cells' (*Blasenzellen* of German authors) are actually connective tissue, or, we might even say, skeletal elements, for they form the bulk of the tunic. They are much like the chorda cells of various invertebrate forms and the notochordal cells of the vertebrate embryo. By far the greater part of the cell is a huge, fluid-filled vacuole. A narrow crescent of cytoplasm can be made out on many of them but is lacking or invisible on others. Of the greatest interest is the almost constant presence in each cell of a small concretion in the vacuole, as in the vacuolate cells of the blood. The concretion in the tunic cells is more often placed peripherally in the vacuole and more frequently somewhat elongated than in the blood-cell. An occasional cell of the tunic shows two or three 'lobes', as though an incomplete fusion of vacuoles had occurred.

Rapkine and Damboviceanu (1925), working with *A. mentula* and using indicators and the micro-injection technique, found the pH of the vacuolate cells and green cells to be much lower than that of the other blood cells (2·8 as compared to 6·0). The pH of the large vesicular elements of the tunic (bladder-cells) was very nearly 2·0.

The subject of the cell population of the ascidian mantle is a complex one. Saint Hilaire (1931), in a description of 31 species of ascidians, has listed no fewer than 14 types of cells of the tunic. In any one species, however, the number of types may not be great, and in *A. atra* we have identified, outside the blood-vessels, only the large 'bladder-cells', blue cells, finely granular amoebocytes (lymphocytes), and coarsely granular amoebocytes (macrophages).

Huus (1937) states that little is known about the extent to which the cells penetrating into the tunic are already differentiated ones or are young cell forms which differentiate into the special cell types only after settling down in the tunic.

The nature of the cells in the ascidian mantle is of particular interest because of the presence of 'animal cellulose' (tunicin) in it. Our knowledge of this substance has been confirmed and amplified by several authors (Saint Hilaire, 1931; Grassman, Zechmeister, Toth, & Stadler, 1933). The cellulose is present in the interstitial matrix of the tunic and seems to permeate the fibres.

Henze thought that there might well be a relation between the vanadium chromogens of the blood-cells and the formation of cellulose—that the vanadium chromogen represents a strong reducing system and might be analogous in this way to the mechanism of the chloroplasts, making use of carbon dioxide. Winterstein (1909) indicated that carbon dioxide is used or bound by the blood, since there is such a small content in the plasma. Florkin (1934) also found less carbon dioxide in the blood than in sea-water.

There is some reason, then, for linking the unusual chemical constitution of the blood with the presence of cellulose in the tunic. A migration of cells from the blood into the substance of the tunic might account for the transport of materials picked up in the blood.

The remaining two types of cells common to the blood of all three species are the green cells and the orange cells. Fulton (1920) speaks of the green cells as ' . . . in a perfectly quiescent state'; while George (1930), speaking of the same species, *A. atra*, calls them 'noticeably amoeboid'.

We have observed motion of the green cells many times. The viscous nature of the green material, in fact, is well seen in the way in which it flows into the pseudopodia. It always maintains a sharply defined interface with the cytoplasm. The green substance blackens readily with osmium tetroxide and disappears if a little 1% acetic acid is added to the preparation. It seems to be a fatty, probably nutritive substance.

Fulton (1920) described the orange cells as showing a 'rapid swimming motion', while George (1930) described them as amoeboid. We have observed the formation of 'pseudopods' or extensions from the cell-body, blunt processes which may project several μ.

In regard to a 'swimming motion', we have watched orange cells many times and have found them often to show a slow but free movement through the fluid while surrounding cells of other types were either entirely quiescent or undergoing amoeboid motion on the glass. On a few of these cells we have made out a long, thin structure which resembles a solitary cilium or flagellum but whether it really is a distinctive 'swimming organ' remains problematical. We have never seen orange cells undergoing progressive amoeboid motion.

The possible relationship between the vacuolate cells and the green cells is a subject on which Fulton (1920) and George (1930) differed completely. According to Fulton, a weak solution of an acid added to a 'fresh smear' of ascidian blood caused some of the vacuolate cells (his Q-2 cells and some of the larger Q-1 cells) to take on a green tinge and to develop lines of division or furrows. In this way, green cells were supposed to develop from the vacuolate cells. Fulton believes that this is the normal course of events—that green cells arise from the vacuolate cells.

Of the supposed transformation of vacuolate into green cells, George (1930, p. 390) said that it 'does not seem tenable in the light of our present knowledge of the structure of ascidian blood cells'.

Our own results, derived from experiments with three different concentrations of acetic acid (table 1), have given no evidence of transformation of vacuolate cells but have shown a rather ready tendency of the contents of the vacuoles to dissolve in a medium more acid than that of the blood. The disappearance of the contents of the vacuole leaves a swollen cell with an 'empty' appearance and with its concretion, now motionless, in a peripheral position.

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The Test of the Ascidian, *Phallusia mammillata*

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With three plates (figs. 4, 7, and 8)

SUMMARY

A mucopolysaccharide, readily removed by mild acid hydrolysis, is associated with cellulose in the test of *Phallusia mammillata* (Cuvier). The mucopolysaccharide is produced in the vacuolated cytoplasm of epidermal cells covering the blood-vessels of the test.

The cellulose of the test appears to be produced by vanadocytes which have migrated into the test matrix through the walls of the test blood-vessels. In the test, the vanadocytes lose their acid and intraglobular osmiophil material and produce processes which extend into the surrounding test matrix. Minute globules and submicroscopic vesicles are present in the cytoplasm surrounding the large polysaccharide-containing globules of such vanadocytes, and also in the processes arising from such cells. The small globules and vesicles appear to arise from the large globules and to be involved in the production of micro-fibrils observed in electron micrographs of the test. The micro-fibrils, which vary from 20 to 40 mμ in diameter and are arranged in the form of an open network, may constitute a cellulose framework within the test.

An area devoid of test matrix occurs usually near each disintegrating vanadocyte. The remnants of each vanadocyte often come to lie in such an area. Capsules—the so-called 'bladder cells' of the test are thereby formed.

Phagocytes occur throughout the test and melanin-containing pigment cells are aggregated around its periphery.

INTRODUCTION

BEFORE the recent discoveries of cellulose fibres in mammalian connective tissue (Hall & others, 1958; Cruise & Jeffery, 1959) and of 'tunicine' in the Pogonophora (Ivanov, 1959), ascidians and their kin comprising the Sub-Phylum Tunicata were believed to be the only animals that synthesized cellulose. Consequently, the ascidian test has been the subject of numerous investigations. For an introduction to the literature on the subject the reader is referred to reviews by Saint-Hilaire (1931) and Pruvot-Fol (1951).

As pointed out by these authors, nitrogenous material, as well as cellulose, occurs in the ascidian test. In the test of the ascidian *Pyura stolonifera* (Heller), a mucopolysaccharide is associated with cellulosic material (Endean, 1955*a*). One of the aims of the present investigation was to ascertain whether the test of *Phallusia mammillata* has a similar chemical constitution.

Several modes of formation of the ascidian test have been postulated (Brien, 1930; Saint-Hilaire, 1931; Pérès, 1945, 1948; Millar, 1951; Pruvot-Fol, 1951). Apart from Pruvot-Fol's suggestion that symbiotic algae may be responsible for cellulose production in ascidians, the concensus of opinion seems to be that epidermal cells play the principal role in test formation.

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However, Brien (1948) points out that the formation of the test coincides with a migration of cells from the haemocoele, and Endean (1955*b*) has shown that certain blood-cells participate in the formation of the test of *Pyura stolonifera*. In view of this, it was decided to investigate the nature and roles of the cells found in the test of *Phallusia mammillata*.

MATERIAL AND METHODS

Adult specimens of *P. mammillata* were obtained from Salcombe, South Devon. They were kept in well-aerated sea-water until required.

Pieces of fresh test were sectioned with a razor and examined by direct, phase-contrast, and polarized-light microscopy. Some sections were dyed supervitally with 0·001% neutral red, 0·001% methylene blue, or 0·1% toluidine blue. Others were exposed to osmium tetroxide vapour before examination.

Frozen sections of material fixed in 5% formalin in sea-water were also examined. Some were dyed with Weigert's haematoxylin and eosin, others with Mann's methyl blue / eosin. Some were subjected to histochemical tests for polysaccharide (PAS reaction), mucopolysaccharide (0·1% toluidine blue, alcian blue 8GS, Hale's 1946 method), protein ($HgCl_2$/BTB), and lipid (Sudan black B).

Paraffin sections of pieces of test fixed in Bouin and Susa were also prepared and dyed with Mallory's or the Azan mixtures, or with pyronin or orcein.

Small pieces of fresh test were fixed with buffered 1% osmium tetroxide (Palade, 1952), embedded in araldite (Glauert & Glauert, 1958) and sectioned on an ultramicrotome of the A. F. Huxley pattern. Sections showing gold interference colours were selected, mounted on formvar-coated grids, and examined in a Phillips electron microscope.

THE STRUCTURE OF THE TEST

The test consists of translucent, gelatinous material bounded externally by a thin brownish layer.

Sections of the test, viewed by direct microscopy, showed the presence of blood-vessels with contained blood-cells, large so-called 'bladder cells', and an apparent variety of smaller cells, all surrounded by hyaline test material (fig. 1). This hyaline material was dyed diffusely by orcein and pyronin but not by Mallory or Azan mixtures. As noted by Saint-Hilaire (1931), no fibrillar structure is apparent in this material when it is viewed by the optical microscope. However, some electron micrographs revealed the presence of a fibrillar network (fig. 4, A). The coarser fibres in this network were about 40 $m\mu$ thick, and were apparently oriented. Smaller fibres about 20 $m\mu$ thick were also present.

The blood-vessels, which ramified through the test, branched extensively and terminated in knob-like bulbils near the outer border of the test. Each blood-vessel possessed a peripheral layer, one cell thick, of epidermal cells.

These cells were, for the most part, disposed in rows running parallel to the long axis of the blood-vessel. In fresh test slices, the cells were observed to possess short conical projections (fig. 2) extending into the surrounding test material, which, in the immediate vicinity of the blood-vessels, was more transparent than elsewhere. Each cell possessed a large ellipsoidal nucleus.

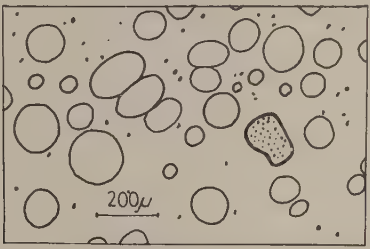

FIG. 1. Section of test of *P. mammillata* showing large 'bladder cells', small test-cells, and a blood-vessel.

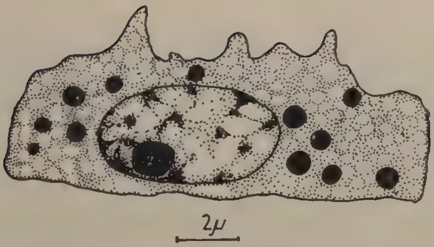

FIG. 2. An epidermal cell from a test blood-vessel fixed with Bouin's fluid and dyed with Azan.

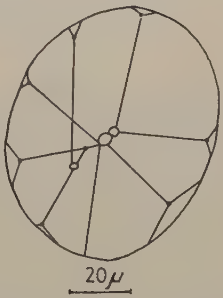

FIG. 3. A 'bladder cell' in optical section, viewed by phase contrast.

The vacuolated cytoplasm of each cell was dyed faintly by the aniline blue component of Mallory and Azan. A few granules, which appeared to be mitochondria, as evidenced by their behaviour to Janus green B used supervitally, were observed in the cytoplasm, particularly near the nucleus. The nucleus possessed a prominent nucleolus, and, in fixed material, a meshwork of basiphil material was apparent.

From the outer border of the test to about three-quarters of the distance to the inner edge of the test, bladder cells were prominent (figs. 1; 3). These cells were circular or oval in cross-section. Just below the outer boundary of the test the spherical cells averaged about 60 μ in diameter but elsewhere they averaged about 120 μ.

A meshwork of very fine fibres or strands which radiated to the periphery of the cells from colourless, poorly refractive, spherical bodies was present in the bladder cells (fig. 3). Some of these bodies were at the limit of resolution, others were as large as 4·5 μ in diameter. The majority were between 1 and 3 μ. At the periphery of the bladder cells the fibres merged with hyaline material, which appeared to be continuous with the substance of the test. Bladder cells that abutted on one another were mutually compressed, presumably because of the turgor of their contained fluid. No nuclei or typical cell organellae were observed. Effectively, the bladder cells are not cells but fluid-filled capsules in each of which there is a taut meshwork of fibre-like processes.

Certain of the cells found in the blood of *P. mammillata* (Endean, 1960) were found in the test. Intact and fragmented pigment cells were prominent in the outermost regions of the test and probably the brownish appearance of this region is due to their presence. Pigment cells were also aggregated in large numbers against the walls of the bulbils. Phagocytes were common in the outer regions of the test and occasionally cells with acidic vacuoles were observed in this region.

Close study of the variously shaped cells found in the hyaline material comprising the bulk of the test revealed that the majority were vanadocytes in varying stages of disintegration.

Many of these test vanadocytes, although giving typical blood vanadocyte reactions to OsO_4, neutral red, and methylene blue, were flattened and often up to 16 μ in diameter (fig. 4, B). Some were irregularly shaped, being branched, with their contained globules disposed along the branches. Also, some had more globules than the vanadocytes of blood were observed to possess. Since many of these globules were small, it seems likely that some globules had fragmented to give smaller ones.

Other vanadocytes were similarly shaped to those just described but only the peripheries of some of their contained globules (or possibly the cytoplasm surrounding the globules) blackened with OsO_4. In other vanadocytes, of similar structural appearance and dimensions, none of the cores of the globules blackened with OsO_4. From the borders of such cells, processes containing granules had arisen and extended out into the surrounding hyaline material (fig. 4, C, D). A further stage was reached in the disintegration of vanadocytes when these were surrounded by a meshwork of fibre-like processes which branched repeatedly. Some of the processes were traced to distances between 70 and 80 μ from the main cell mass before they became so fine that they were lost to view in the hyaline substance of the test. Along the length of the processes, and particularly at the points of branching, minute granules, some

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Fig. 4 (plate). A, electron micrograph of section through test showing network of microfibrils. Part of the distal region of a fibre-like process (*p*) is visible.

B, photomicrograph of a test vanadocyte.

C, photomicrograph of a test vanadocyte which has begun to produce fibre-like processes.

D, photomicrograph of a partially disintegrated test vanadocyte showing some of the fibre-like processes produced. All photomicrographs are of cells in test sections fixed with OsO_4 vapour.

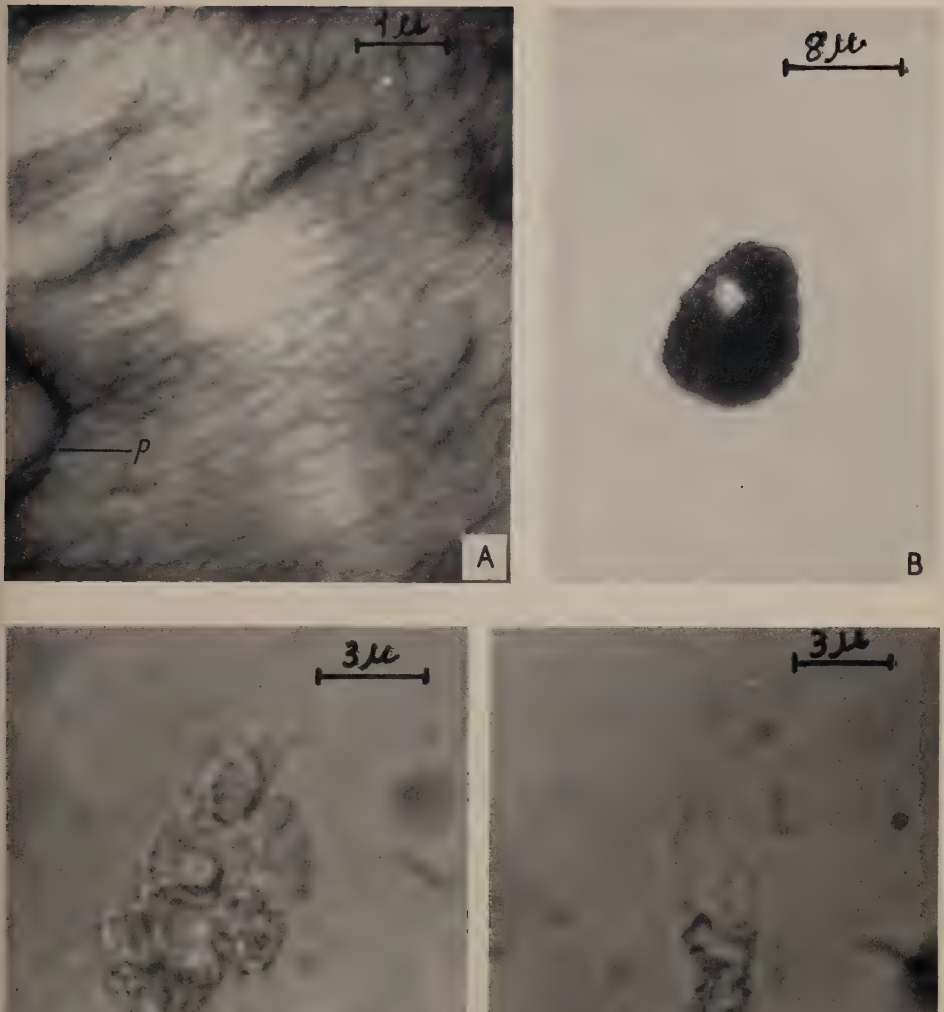

FIG. 4

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at the limit of resolution, others ranging up to 0·5 μ in diameter, were in evidence (fig. 5).

There was a progressive decrease in the size of the central mass and a reduction in the number of globules as the vanadocytes disintegrated further. As the central mass of each disintegrating vanadocyte decreased in volume the nucleus of each cell became progressively more indistinct and was usually no longer visible when the central mass was less than 5 μ in diameter. The globules left in the reduced central mass were often refractory to dyes and appeared as mere 'ghosts'.

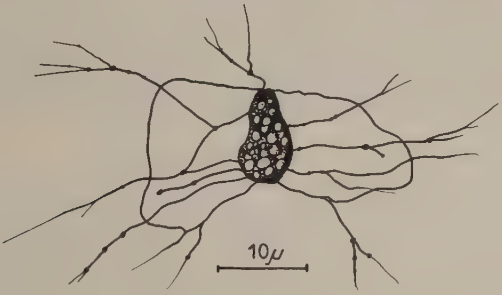

FIG. 5. A test vanadocyte in optical section, showing granule-containing processes crossing areas devoid of test substance in the immediate vicinity of the cell. The cell has been fixed with OsO_4 vapour.

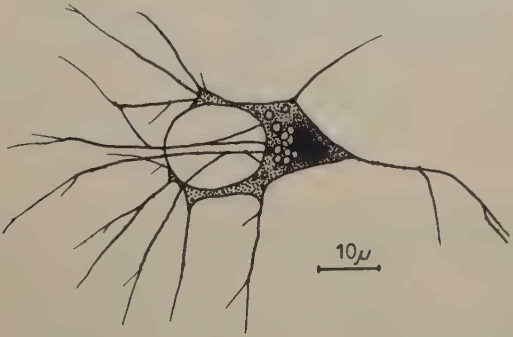

FIG. 6. A partially disintegrated test vanadocyte in optical section. An area, oval in outline and devoid of test material, is almost enclosed by cytoplasmic projections. Granule-containing processes have arisen from the cytoplasm of the cell, which has been dyed supervitally with 0·1% toluidine blue. The central mass consists of globules dyed blue with the dye and of other globules which are not dyed, and which appear to be 'ghosts'.

Frequently an area (or areas) devoid of test matrix was observed in the immediate vicinity of a disintegrating vanadocyte. Sometimes the clear area surrounded the central mass of such a vanadocyte, sometimes it was to one side (fig. 6). Processes containing granules crossed the clear areas and extended into the surrounding test matrix. It was evident that small test capsules arise from such disintegrating vanadocytes and that these capsules each contain the remnants of a vanadocyte enclosed in a fluid-filled cavity. The taut fibres inside these capsules are the stretched proximal regions of fibre-like processes. Larger capsules presumably result from a further accumulation of fluid.

In some cases vanadocytes had been disrupted and their contained globules had separated one from another. Each globule had acted as a centre from which granule-containing processes had originated. There was no dissolution of test material in the immediate vicinity of these isolated globules.

Small wedge-shaped pieces were cut from the peripheral region of the test of living specimens of *P. mammillata* and the animals were then returned to a tank of sea-water. After 72 h noticeable regeneration of the test had occurred. Large numbers of cells appeared to have aggregated in the regenerated regions. Some were typical vanadocytes and many were disintegrating vanadocytes surrounded by processes. Most, however, proved to be globules which had separated from their parent vanadocytes and were surrounded by interlacing granule-containing processes. These globules gave a purplish-red colour with Mann's methyl blue / eosin which contrasted with the yellow colour given by the globules of typical vanadocytes in the test and in adjacent blood-vessels. Some vanadocytes appeared to have been fixed while in transit through the walls of these blood-vessels.

In electron micrographs of sections of vanadocytes from the test, globules were easily recognized. Some globules (fig. 7, A) contained electron-dense material which appeared irregularly dispersed. Such globules probably correspond with those observed, under the optical microscope, to have blackened with OsO_4. The globules of other vanadocytes (fig. 7, B) contained only small amounts of material of high electron density, and, in still other vanadocytes (figs. 7, C; 8, A), only material of low electron density was present in the globules. However, electron-dense material was present around the peripheries of the globules and these vanadocytes correspond with those observed under the optical microscope to have blackened with OsO_4 only around the peripheries of the globules. In electron micrographs of sections of these cells minute vesicles (averaging about 80 mμ in diameter) were present in the cytoplasm, particularly near the cell boundaries. Similar vesicles, some only partially differentiated, were apparent in and around the edges of some globules. This suggests that the vesicles are derived from the globules and that each vesicle encloses part of the material of low electron density found in the globules.

FIG. 7 (plate). A, electron micrograph of a section through part of a test vanadocyte showing two large globules and portions of others containing electron-dense material.

B, electron micrograph of a section through part of a test vanadocyte which shows little electron-dense material within its globules. Vesicles (*v*) are present below the cell surface and around the periphery of a globule.

C, electron micrograph of a section through a test vanadocyte which possesses globules almost devoid of electron-dense material.

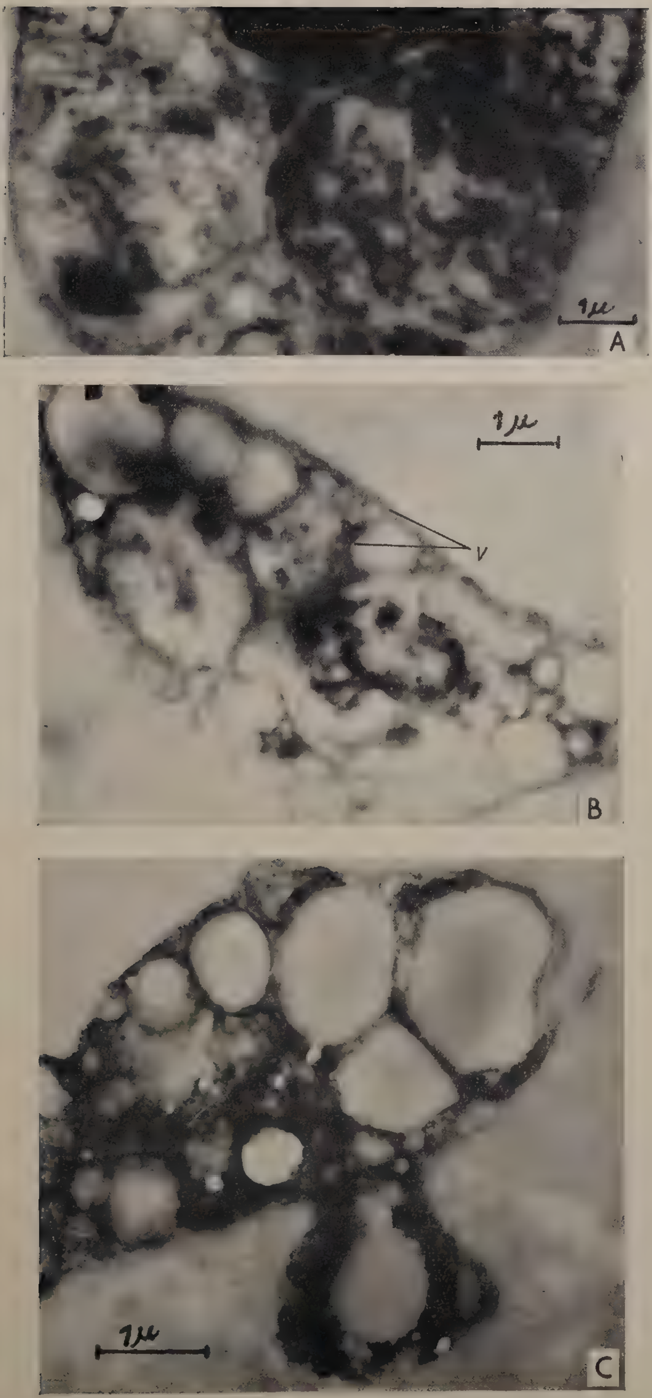

Fig. 7

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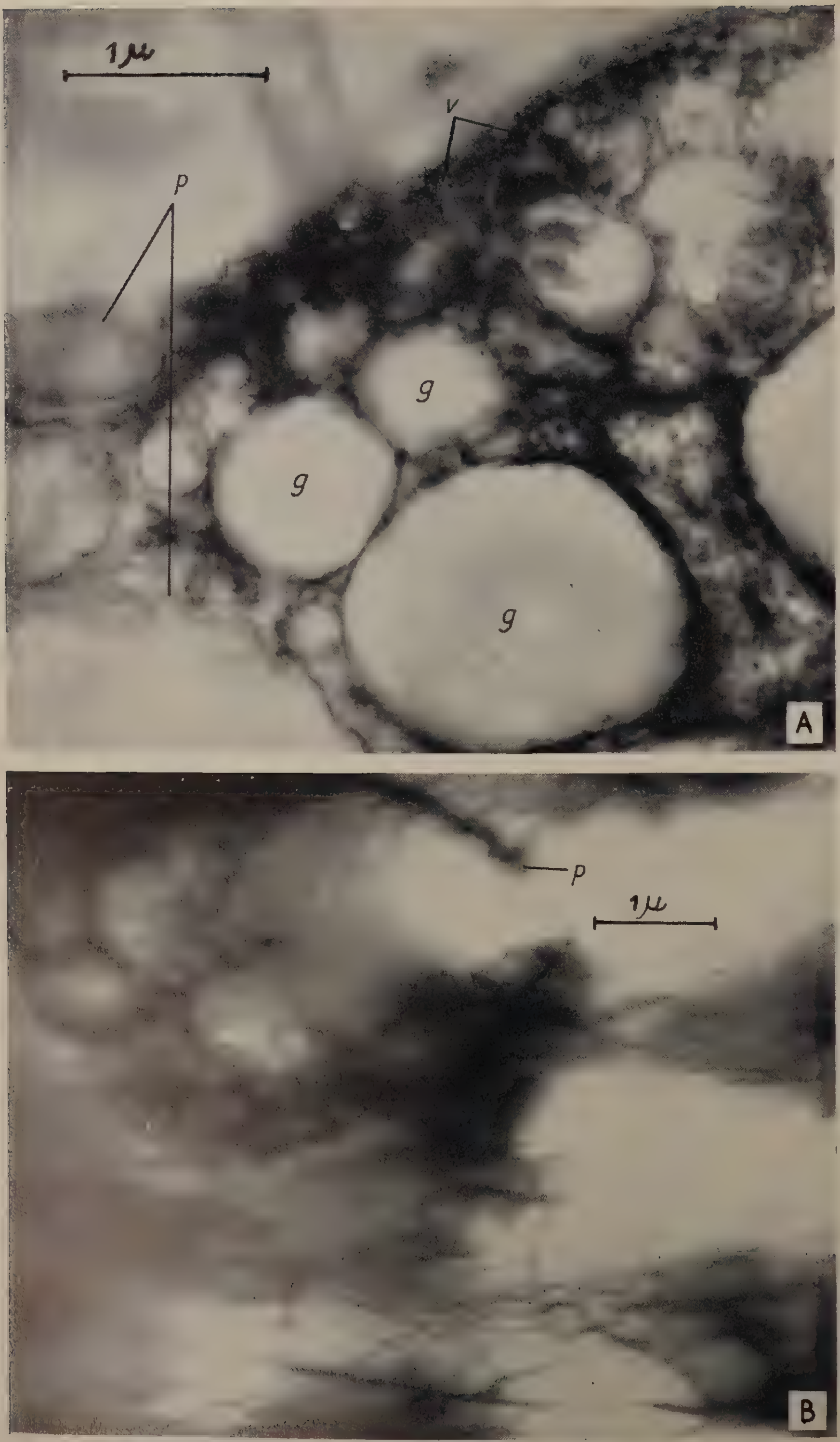

FIG. 8

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Some of the larger globules appeared to be in the process of breaking up to give smaller globules.

In electron micrographs of some sections of test vanadocytes, projections of cytoplasm containing globules of various sizes, and vesicles, were noted. Many of the cytoplasmic projections were attenuated distally. Although the vesicles and most of the globules present in the processes would be too small o be visible with the optical microscope, a few larger globules, spaced at rregular intervals along the processes, and situated at or near points of branchng of the processes, would be visible. These larger globules probably correpond with the 'granules' of 'granule-containing' fibre-like processes seen with he optical microscope and described on p. 110.

In those electron micrographs where micro-fibrils were apparent, the ibrils were continuous with the surfaces of vanadocytes and their processes. This suggests that the micro-fibrils are derived from vanadocytes. Indeed, micro-fibrils from 20 to 40 mμ in diameter appeared to arise directly from the urfaces of cytoplasmic projections of vanadocytes (fig. 8, B) and to have hredded from the surface of each fibre-like process (figs. 4, A; 8, B).

Areas which seemed devoid of fibrillar material were often noted in electron micrographs of sections of vanadocytes in the immediate vicinity of vanadocytes. These areas were sometimes crossed by processes from vanadocytes nd these areas seemed to correspond with the clear areas observed with the optical microscope near disintegrating test vanadocytes (p. 111).

HISTOCHEMISTRY OF THE TEST

The intercellular matrix. This gave a positive PAS reaction but did not ontain protein detectable by the $HgCl_2$/BTB reagent. With toluidine blue he matrix gave strong γ-metachromasy which was most intense in the immediate vicinity of blood-vessels. It gave a deep blue colour with alcian blue GS and a strong blue coloration with Hale's acid mucopolysaccharide test.

Testicular hyaluronidase at 0·1% did not remove the PAS-positive metahromatic material. However, if formalin-fixed sections were heated at 98° C with N H_2SO_4 or N HCl for 10 min the metachromatic material was completely removed. The test sections were not visibly affected by the treatment with acid. Subsequently they gave a faint PAS reaction and no reaction for rotein.

The above results indicate that the intercellular matrix of the test contains cid mucopolysaccharide which is not hyaluronic acid and a frame substance

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FIG. 8 (plate). A, electron micrograph of a section through part of a test vanadocyte, showing lobules (*g*), vesicles (*v*), and the base of a granule-containing process (*p*), the cytoplasm of which is continuous with that of the cell.

B, electron micrograph of a section through a portion of a disintegrating vanadocyte containing globules and vesicles. Part of a fibre-like process (*p*) is shown. Micro-fibrils appear to rise directly from the surfaces of the cell and process.

which does not appear to be proteinaceous but which may contain polysaccharide.

The epidermal cells of the blood-vessels. The vacuolated cytoplasm of these cells was PAS-positive and showed strong γ-metachromasy with toluidine blue. A positive result was obtained for acid mucopolysaccharide with Hale's (1946) method. Treatment with N HCl for 10 min at 98° C removed the mucopolysaccharide from the cells. No PAS reaction was then given by these cells.

The test cells. The histochemistry of pigment cells, phagocytes, cells with acidic vacuoles, and vanadocytes, has been described in an earlier paper (Endean, 1960).

Some of the flattened vanadocytes of the test reacted with the dyes and histochemical reagents used in this investigation in the same way as typical blood vanadocytes. The globules of such vanadocytes blackened with OsO_4. However, as noted earlier, the globules of vanadocytes which were producing processes had lost the ability to blacken with OsO_4. Such globules appeared to have lost part or all of their acid also, for they became deep blue (instead of yellow) with $HgCl_2$/BTB and pink (instead of deep red) with neutral red. In fixed material, they were amphoteric in their behaviour towards dyes. Thus they were dyed purple or red by Mann's methyl blue / eosin, red by Azan, orange by Mallory, and red by pyronin. It was thought that the fixatives used may have caused a release of acid from the globules, but vanadocytes fixed in the blood-vessels of the same sections were strongly basiphil, as are the typical vanadocytes of the blood.

Vanadocytes that were producing fibrous processes were strongly PAS-positive, as were their processes and the granules on these processes. Although the vanadocytes gave a deep blue colour with toluidine blue, the processes and their granules were blue to purplish, near the vanadocytes, and purplish red to red distally. If a vacuole or vacuoles were present in the vicinity of a disintegrating vanadocyte they were coloured a purplish red by the dye.

The aniline blue component of Mallory and Azan dyed the granule-containing processes blue. These processes were dyed a faint blue by $HgCl_2$/BTB.

Proteinaceous material detectable with $HgCl_2$/BTB gradually disappeared from the disintegrating vanadocytes and none was present in the test capsules. Acid was absent from the capsules, but a mucopolysaccharide, which gave similar histochemical reactions to the mucopolysaccharide of the test, was present.

CHEMISTRY OF THE TEST

Weighed samples of gelatinous test were heated at 104° C in an oven unti constant weight was obtained. The percentage loss in weight averaged 94·1 % indicating that the test has a remarkably high water-content.

Samples of the test after water, water-soluble, and acetone-soluble materia had been removed were subjected to hydrolysis with 2 N H_2SO_4 for 8 h a

100° C. There was little apparent disintegration of the test material and the atter was filtered off and retained. The hydrolysate, in each case, was neutraized with $BaCO_3$ and centrifuged, and the volume of the supernatants reduced by evaporation under reduced pressure. The concentrated supernatants gave a strong Molisch reaction and reduced Benedict's solution. They gave a positive result when tested for hexosamine following the procedure of Elson and Morgan (1933).

Samples of the test residue, washed with distilled water, were placed in contact with 80% H_2SO_4 for 3 days. The acid was then diluted to 2 N H_2SO_4 and the material hydrolysed by heating at 100° C for 16 h. The hydrolysates were then neutralized with $BaCO_3$ and centrifuged, and the volume of the upernatants reduced in each case by evaporation under reduced pressure. A trong Molisch reaction was given by the supernatants and these also reduced Benedict's solution.

A drop of each supernatant was placed on a filter paper strip and chromatograms run with ethyl acetate / pyridine / water as solvent. The papers were dried and sprayed with *p*-anisidine. A single spot was given in each case and hese spots corresponded with those given by drops of pure glucose run at he same time as reference markers.

Discussion

Over 90% of the test of *P. mammillata* is composed of water. Part of the organic material present gives histochemical reactions indicative of acid mucopolysaccharide. This material can be readily removed by subjecting the est to mild hydrolysis, and hexosamine is present in the hydrolysate. The esidual insoluble material contains no protein and, on hydrolysis, yields glucose as the only sugar. It appears to be cellulose, which has been found epeatedly in the tests of ascidians since its occurrence there was first reported by Schmidt in 1845.

Some electron micrographs of the test revealed the presence of an open meshwork of micro-fibrils. It is perhaps significant that the diameters of the micro-fibrils correspond with those recorded for cellulose micro-fibrils in plant fibres (Mühlethaler, 1949). Other sections of the test revealed no traces f micro-fibrils and it is possible that those observed were artifacts or that micro-fibrils were present only in certain regions of the test. However, it is also possible that the apparent absence of micro-fibrils in some test sections was due to poor penetration of the OsO_4 fixative. Prolonged periods in histoogical fixatives were necessary in order to ensure that pieces of the test were uitably fixed for examination with the optical microscope.

In a previous paper (Endean, 1960) it was shown that the spherical blood vanadocytes possess conspicuous globules in which polysaccharide is synhesized in an acid medium in the presence of a vanadium chromogen and hat these cells must leave the blood at some stage. It has now been shown that ully developed vanadocytes migrate into the test substance, where they give ise to fibre-like processes. Acid as well as osmiophil material (believed to be the vanadium chromogen) are soon lost from the globules, and the cells disintegrate. Study of electron micrographs suggests that the residual intra-globular material is released into the surrounding cytoplasm in small vesicles. Since this residual material, as evidenced by histochemical tests, is composed chiefly of polysaccharide, it is probable that the vesicles contain polysaccharide. The whole cell, including processes, is strongly PAS-positive at this stage but only the distal parts of the fibre-like processes exhibit metachromasy with toluidine blue. Probably this is due to the processes becoming associated with the mucopolysaccharide of the test.

The test mucopolysaccharide appears to be produced in the vacuolated cytoplasm of the epidermal cells lining the blood-vessels of the test. Histochemical tests show that mucopolysaccharide is present in the cytoplasm of these cells and is more concentrated in the test matrix in the immediate vicinity of these cells than elsewhere in the test. After treatment with dilute acid no residual mucopolysaccharide could be detected in these cells, whereas the polysaccharide material in vanadocytes resists the action of dilute acid (Endean, 1960). These observations militate against the epidermal cells themselves being involved in the production of cellulosic material but suggest strongly that the vanadocytes are so involved. Also, the micro-fibrils of the test appear to arise directly from the surfaces of vanadocytes and their processes. It seems likely that the sub-microscopic vesicles found just below these surfaces are involved in the formation of fibrils.

Growth of the test appears to be by intussusception—newly formed micro-fibrils interlacing with those already present. An increase in test volume might also result from an increase in the number of fluid-filled capsules in the test. With respect to the formation of these capsules, it may be of importance that the globules of vanadocytes contain free H_2SO_4, the concentration of which has been calculated by Webb (1939) to be about 1·8 N. As the test mucopolysaccharide is readily removed from sections of the test by N H_2SO_4, mucopolysaccharide molecules in the neighbourhood of disintegrating vanadocytes would be affected by acid released from vanadocyte globules. This may affect the stability of the test matrix in the immediate vicinity of such vanadocytes and result in the formation of the clear areas frequently observed in such regions. Why these areas devoid of test substance should subsequently become spherical, accumulate test fluid, and increase in size, thereby forming typical test capsules, is not clear. However, the dissolution of the test matrix in the immediate vicinity of vanadocytes would facilitate the penetration of vanadocyte processes into this matrix.

As well as an outflow of vanadocytes from the blood-stream there must be an outflow of phagocytes and fully differentiated pigment cells (Endean, 1960). Phagocytes were common in the test, where they appear to be responsible for conveying necrotic cells and foreign material to the exterior. Pigment cells formed a covering around the periphery of the test, and, since they contain melanin, it is probable that they afford a protection against ultra-violet light.

This paper embodies the results of part of the research work carried out in 1958–9 during the tenure of a Nuffield Dominion Travelling Fellowship. The assistance, financial and otherwise, received from the Nuffield Foundation is gratefully acknowledged. The author wishes to thank Professor J. E. Harris of the Zoology Department, University of Bristol (where the bulk of this research was carried out), for providing research facilities and for reading and discussing the manuscript. The author also wishes to thank Dr. F. S. Russell of the Marine Laboratory, Plymouth, for providing research facilities at Plymouth. The assistance in the use of the electron microscope provided by Miss E. Harris of the Physics Department, University of Bristol, is gratefully acknowledged.

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A Morphological and Histochemical Study of Oogenesis in the Gall-fly *Cynips folii*

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With five plates (figs. 1–5)

SUMMARY

In the ovariole of *Cynips*, cell groups that have arisen in the initial period of oogenesis, presumably from one gonial cell, differentiate into the oocyte and 12 nurse cells (which constitute the nutritive chamber). The oocyte remains connected with the nurse cells by a strand of cytoplasm; the whole group acts as a physiological unit till the completion of vitellogenesis. Ribonucleoproteins are synthesized in the nurse cells and transmitted to the oocyte. The latter produces no detectable RNA.

There is no morphological evidence of any interchange of material between the nuclei or nucleoli and the cytoplasm of the oocyte or nurse cells.

Protein yolk components appear in the oocyte around the RNA inflow from the nurse cells. They associate with mucopolysaccharides, which appear in the same territory, to form carbohydrate-protein complexes. The synthesis of lipid yolk starts in the posterior part of the undifferentiated cell group and occurs subsequently only in the oocyte.

Nurse cells never penetrate inside the oocyte in normal undamaged ovarioles. They shrink and disintegrate outside the ovariole after completion of their life-cycle.

The anterior part of the oocyte develops into the egg pedicel with a terminal extension in which most of the glycogen of the cell is accumulated; the protein spheres in this extension are different from the protein yolk of the main part of the oocyte. The precursors of these protein spheres arise in the cytoplasm of the common cell group before the onset of yolk formation.

The function of the follicular epithelium is secretion of the egg envelope, which is of epicuticular nature. The penetration of material from the haemolymph to the oocyte occurs mainly through the cells of the nutritive chamber. The contribution of the follicular epithelium to this process seems to be of minor importance, at any rate in the early stages.

INTRODUCTION

IN the ovariole of *Cynips*, as in that of all polytrophic ovarioles of insects, the oocytes alternate regularly with the groups of nurse cells that form the nutritive chambers (figs. 3, C; 4, A). In the initial stage of vitellogenesis basiphil material streams from the chamber into the oocyte (fig. 5, B). The mature egg is prolonged into a pedicel with a terminal extension (fig. 1, D).

In the present paper an attempt is made to show how the ovariole attains its final structure and to indicate the nature of the basiphil inflow from the nurse cells.

MATERIAL AND METHODS

Ovaries of the agamic autumn generation of *Cynips folii* females were used as material, from the last larval stage, shortly before pupation, onwards. For

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study of the differentiation of the early gonad, whole larvae were fixed. Yolk formation was followed in ovaries of pupae and adult insects after they had been isolated from the dissected abdomen in a drop of 0·75% NaCl solution. The material was fixed in Zenker (without acetic acid), Carnoy, and Champy, in osmium / potassium dichromate / mercuric chloride, and in neutralized 10% formalin. The material fixed in Zenker proved most satisfactory but was not suitable for the elimination of RNA with ribonuclease or perchloric acid, and had to be controlled by material fixed in Carnoy. Material embedded in paraffin was sectioned at 6 μ. Material was cut on the freezing microtome (after formaldehyde fixation) at 10 μ.

The histochemical results are based on the following tests: Feulgen reaction; pyronin / methyl green of Brachet; panoptic of Pappenheim; PAS after McManus; Schiff–Bauer's fuchsine; iodine of Langhans; Mazia's bromophenol blue; ninhydrine after Ruheman–Abderhalden and after Iusuma–Isikava; xanthoprotein reaction; trichrome stain of Himes–Moriber; Nile blue; Sudan III; Sudan black. The following were also used: haematoxylin/eosin; methyl blue / eosin of Mann; alcian blue; neutral red and trypan blue as vital dyes.

RESULTS

Morphogenesis of the ovariole

In the earliest stage observed (larva before pupation) the ovary is an elongate pyramid, tapering anteriorly (fig. 1, A). It contains cells in the prophase of meiosis. The nuclei in the narrow anterior part show thin leptotene threads and a big acidophil homogenous nucleolus; the cytoplasm is basiphil. Cells are not clearly separate but form more or less distinct syncytial groups. They are alike: no distinction between future oocyte and nurse cells is observed at this stage. In the next zone of synapses the polarized chromosomes are intensely Feulgen-positive, and cytoplasmic basiphilia abruptly decreases. Cells in the broad posterior zone of the ovary start growing. These cells are in the pachytene stage. The basiphilia of their cytoplasm increases. The oocytes soon become distinguishable from the nurse cells by becoming less strongly Feulgen-positive in their nuclei.

At this stage the number of cells in each group may be determined. There are 12 nurse cells and one oocyte in each group. The constant number suggests their origin from one primitive germ cell, which, after two successive divisions, produced 4 cells; 3 of them divided twice giving 12 nurse cells,

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FIG. 1 (plate). A, ovary of the last larval stage, in longitudinal section. (Zenker; Feulgen, light green.)

B, ovary at slightly later stage than A, in transverse section. Small amoebocytes are seen among the cell groups. The migration of haemocytes is seen. (Zenker; Feulgen, light green.)

C, ovary of early (white) pupa, in longitudinal section. The upper cell-groups are undifferentiated. The shifting of the oocyte is seen in the lower groups. The distribution of RNA is shown. (Zenker, Pappenheim.)

D, Whole mature egg, before oviposition. (Living, unstained.)

E, ovary of greyish-white pupa (older than C), in longitudinal section. Formation of follicles. (Zenker; Feulgen, light green.)

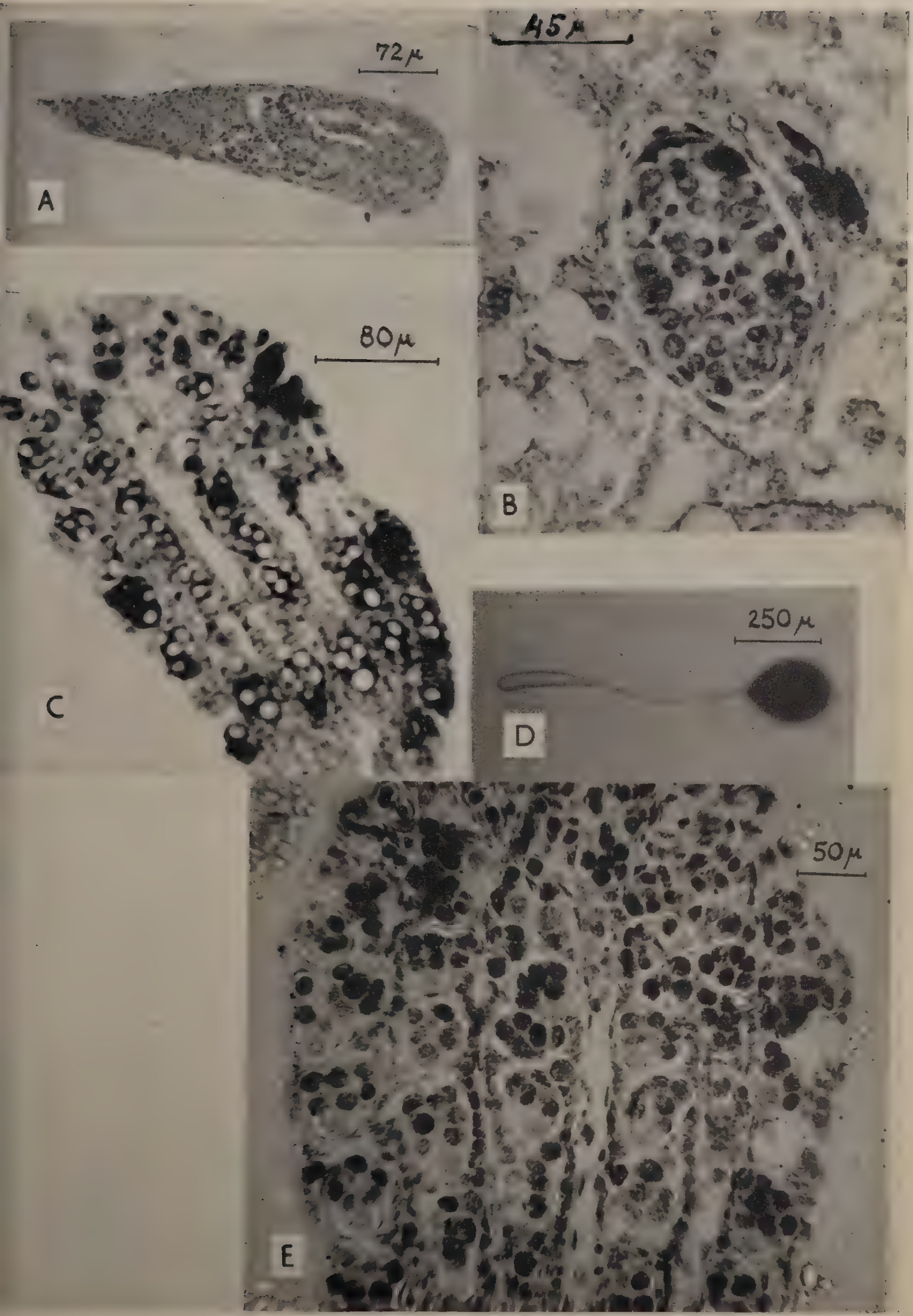

FIG. 1

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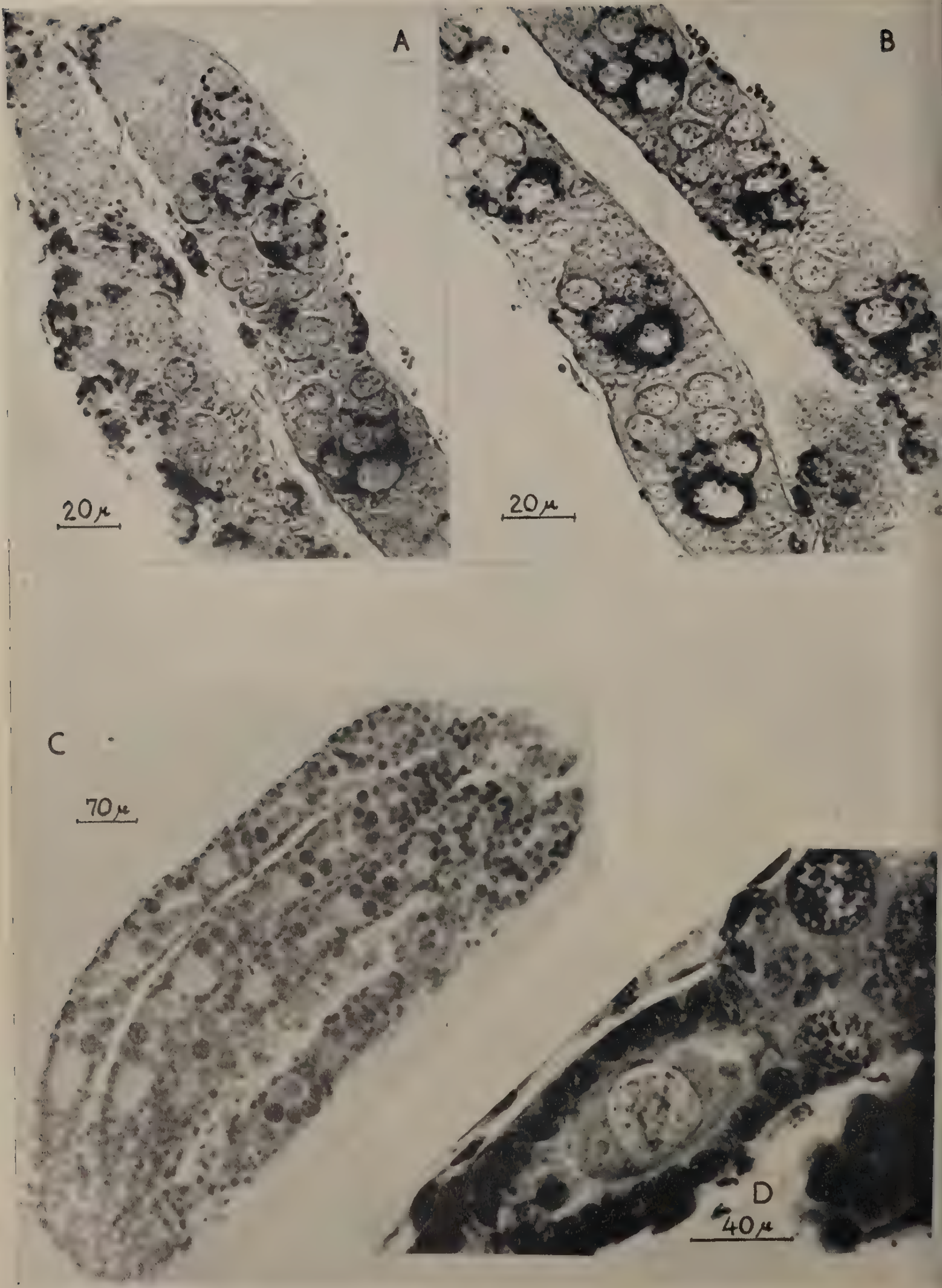

Fig. 2

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and the fourth cell—the oocyte—remained undivided. The divisions probably occurred at a much earlier stage and were not observed in this study. The conclusions of Paulcke (1901) are followed, who postulated it for the *Apis* ovary. The common origin of the nurse cells and the oocyte is important, because the 13 cells retain their continuity and act as a physiological unit till the conclusion of vitellogenesis.

Haemocytes from the surrounding haemolymph migrate and adhere to the surface of the gonad during the early period of its formation, and produce an envelope of short fusiform cells (fig. 1, B). The envelope is not complete, for there are gaps between its cells. From the inner aspect of the envelope, cells migrate into the gonad rudiment, adhere to the cell groups, and organize them into longitudinal cords—the rudiments of the ovarioles. The primitive gonad envelope disappears finally as a uniform structure enclosing the whole organ when all its cells have applied themselves to the surface of the separate ovarioles. Before this occurred, new haemocytes continued to join the gonad surface.

The follicular epithelium is formed by the ingrowth of epithelial cells from an agglomeration in the oviduct region. The cells become wedged between the cords of cells and the external membrane of fusiform cells (fig. 1, E).

Further development of the ovariole concerns the differentiation of the cell groups: the oocyte and nurse cells. In the posterior (oldest) cell group of the ovariole the oocyte shifts backwards (figs. 1, E; 2, A–C). Its nucleus is at this stage Feulgen-negative. A small, spherical, acidophil body appears in the ooplasm.

The oocyte grows faster than the nurse cells. Its connexion with the nutritive chamber becomes gradually reduced to a narrow strand of cytoplasm after the complete formation of the follicle (figs. 3, B; 5, B, D), but the continuity of the former group of cells is not severed.

The follicular epithelium is organized in the course of differentiation of the cell group. Originally the epithelium covers the cell group uniformly as a continuous layer of a few cells (figs. 2, C; 3, A), coating the nutritive chamber as well as the oocyte. When the oocyte has begun to grow, the epithelium differentiates in three respects.

(1) Cells at the surface of the oocyte multiply rapidly, and the follicle enlarges with growth of the oocyte. With the onset of vitellogenesis divisions of the follicular cells cease and further extension of the follicle occurs by growth and flattening of its cells.

(2) The epithelial cells on the wall of the nutritive chamber neither divide

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FIG. 2 (plate). A, anterior, and B, posterior part of the same two ovarioles, of the same age as in fig. 1, E; longitudinal sections. Initial formation of lipids in the cell groups. (Osmium / potassium dichromate / mercuric chloride; unstained.)

C, ovary of an older (brown) pupa, showing differentiation of anterior (upper) cell groups and growth of posterior groups. Differentiation of follicles. Longitudinal section. (Zenker; Feulgen, light green.)

D, oocyte of a black pupa, showing protein bodies in polar position, and migration of lipocytes on the surface of the epithelium. Longitudinal section. (Zenker; Feulgen, light green.)

nor grow, and never form a continuous layer. They remain wedged between adjacent nurse cells (fig. 3, D).

(3) The cells covering the posterior aspect of the nutritive chamber (interfollicular epithelium) form a compact accumulation of small cells supporting the cytoplasmic connexion of the oocyte with the nutritive chamber. In this way the lateral aspect of the chamber remains without any continuous coating except the thin membrane of fusiform cells originating (as stated above) from the primitive envelope of the gonad rudiment. This membrane is the only tissue separating the nurse cells from the haemolymph.

The growth of the oocyte proceeds quickly after its separation from the group of nurse cells. The ooplasm gradually becomes filled with yolk elements. The oocyte elongates and becomes asymmetric since its anterior part is on one side transformed into a long, thin process adhering to the surface of the oocyte that lies next in front (fig. 4, C). As the result of this deformation the oocyte is gradually displaced from its axial position in the ovariole by distortion of its anterior projection, which is the rudiment of the egg pedicel (figs. 1, D; 4, C).

The anterior apex of the ovariole is prolonged into a terminal filament of narrow cells. These seem to originate from germ cells differentiated at an early stage. Their cycle was not followed in this study.

The anterior terminal cell groups of the ovariole often degenerate before being differentiated. As a result, the number of mature eggs in the ovariole is variable and lower than the number of initial groups. The mature ovariole contains 6 to 8 eggs, loosely connected with one another by their curved pedicels (fig. 4, B). All the eggs of the ovariole mature simultaneously, although the development of the anterior ones was delayed in the early stages.

No regressive changes are manifest in the nutritive chamber cells before they have lost their connexion with the oocyte after completion of yolk synthesis. They then shrink and disintegrate outside the ovariole.

In normal undamaged ovarioles of *Cynips*, the nuclei of the nurse cells are never absorbed by the oocyte. The cycle of these cells was followed and no changes were observed except in a few ovarioles, in which nurse-cell nuclei were found inside the ooplasm; but those ovarioles were elongated and obviously damaged.

The follicular epithelium and the terminal filament regress and disappear, like the nurse cells, before the oocyte matures.

FIG. 3 (plate). A, part of the ovariole seen in fig. 2, C, showing protein bodies and migration of lipocytes. Longitudinal section. (Zenker; Feulgen, light green.)

B, part of ovariole of imago soon after metamorphosis, in longitudinal section, showing protein bodies and lipocytes. (Same treatment as fig. 2, A, B.)

C, part of ovariole of imago soon after metamorphosis, showing rings of lipocytes in the interfollicular constrictions. (Unfixed, unstained, examined whole.)

D, oocyte of a black pupa, showing protein bodies and the superficial layer of ooplasm. Longitudinal section. (Zenker; Pappenheim.)

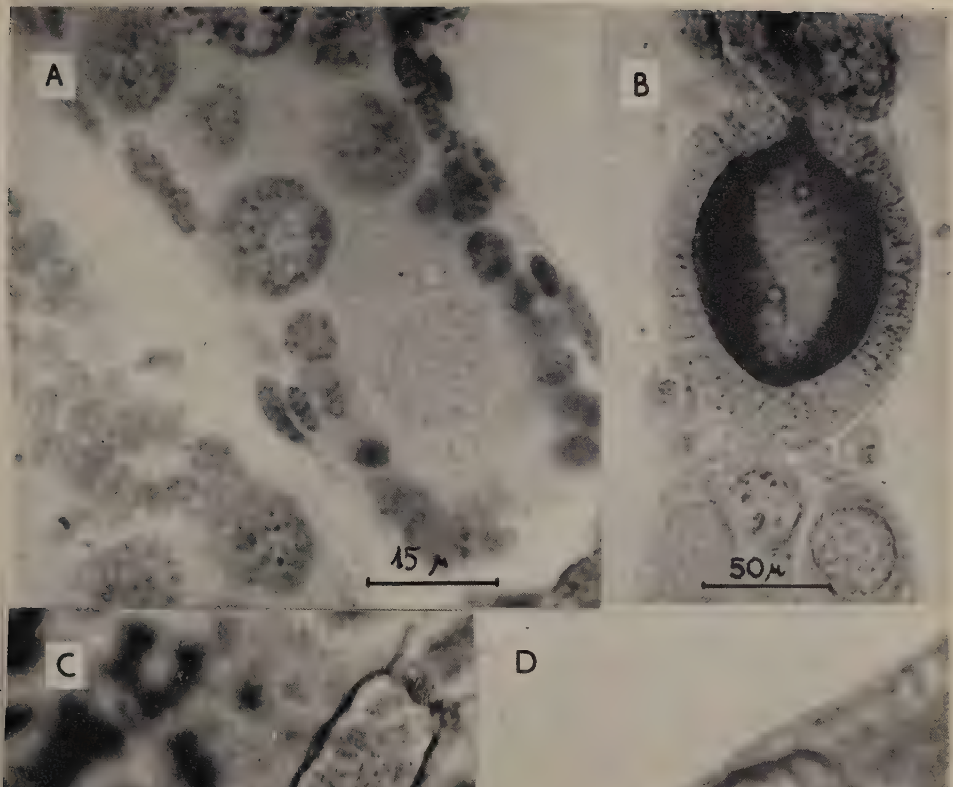

FIG. 3

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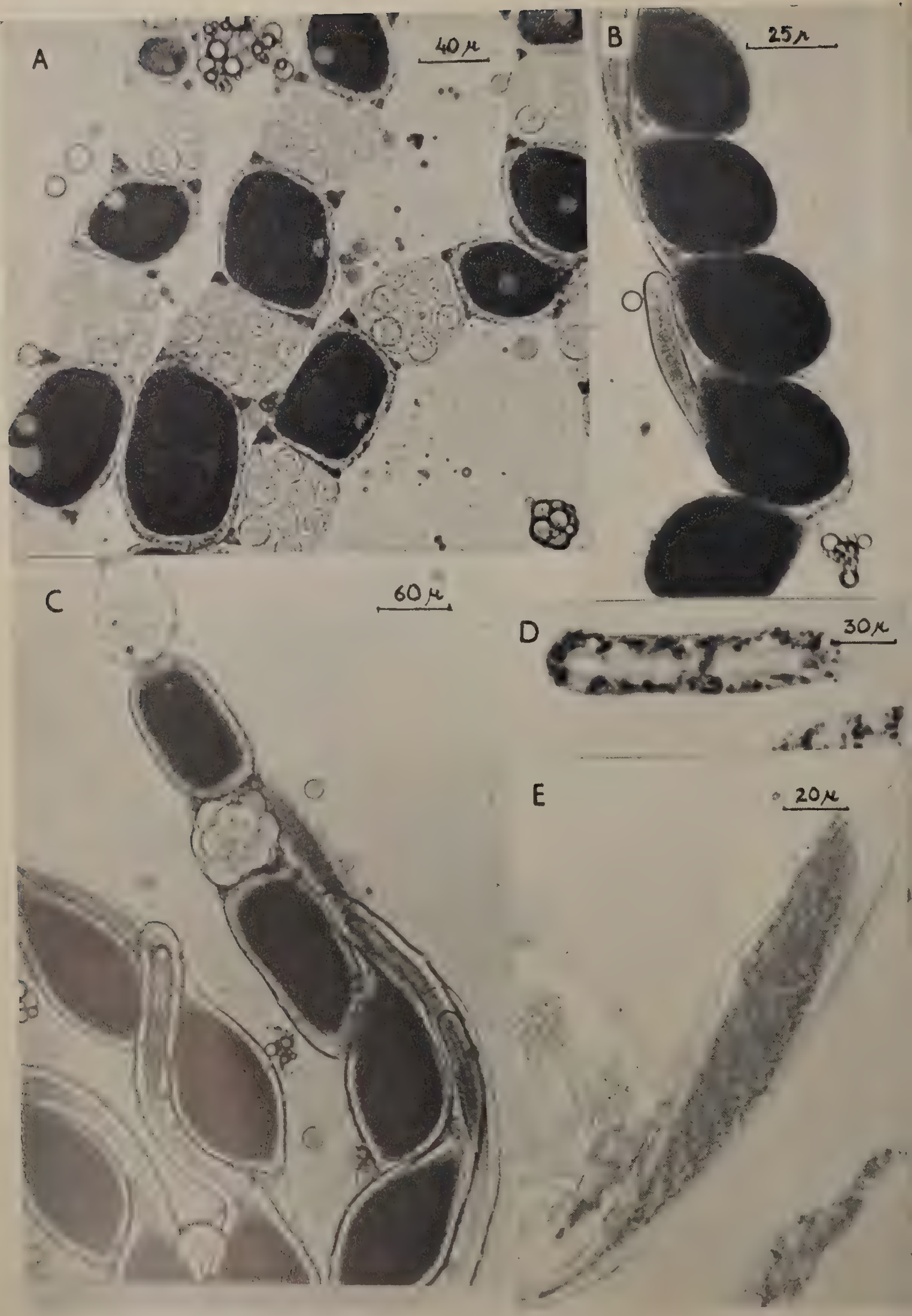

Fig. 4

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Vitellogenesis

The growth of the nurse cells and the changes in their nuclear structure occur simultaneously with the differentiation of the group. The chromosomes, still in the pachytene stage, disintegrate into fragments regularly spaced in the nucleus (fig. 3, A). No accumulation of nuclear granules near the nucleolus or the nuclear membrane was observed, nor was there any evidence of the passage of material from the nucleus or nucleolus to the cytoplasm. The nucleoli (one or two) of the nurse cells are homogeneous and intensely acidophil, and reveal positive reactions for proteins. RNA is localized in their superficial layer. Irregular acidophil PAS-positive bodies are scattered in the nucleoplasm. They are absent in the nuclei of the young chamber, appearing only when RNA begins to flow into the oocyte. They represent a carbohydrate-protein complex, presumably connected with the synthetic activity of nurse-cell nuclei. No similar structures have been observed in the oocyte nucleus. The homogenous cytoplasm of the nurse cells keeps its high RNA content till the conclusion of yolk formation.

The oocyte. A thin acidophil layer differentiates on the periphery of the oocyte (fig. 3, D) as soon as it has separated from the common cell group, when the follicle is being organized. It extends as far as the base of the connective strand. The peripheral layer is homogeneous and glassy in appearance, free of RNA and of yolk elements. In the nurse cells a similar layer is never formed.

In the initial stage of growth and yolk formation, 3 processes occur in the ooplasm: formation of lipid yolk, gradual decrease of RNA content, and inflow of basiphil material from the nutritive chamber.

The lipid components of the yolk appear in the cytoplasm of the common cell group as soon as the oocyte nucleus has begun to be distinguishable. They first arise in the posterior part of the cytoplasm of the group, in the perinuclear zone of the oocyte, and adjacent nurse cells (fig. 2, A, B). When growth and shifting backwards of the oocyte begin, the lipid inclusions migrate with all the posterior cytoplasm of the cell group, and pass inside the oocyte (fig. 2, A, B). A small amount of lipid remains for some time in the posterior nurse cells (fig. 5, D), but finally is also transported into the oocyte with the flow of RNA. The nutritive chamber contains no lipid inclusions when vitellogenesis is at an advanced stage. In the growing oocyte the primary lipid components

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FIG. 4 (plate). A, fragments of fresh ovarioles from an imago of the same age as fig. 5, B. Two lipocytes are seen in each constriction; the rest are out of focus. (Whole preparations; unstained.)

B, mature ovariole, fresh. The eggs are on the right, their pedicels on the left. (Whole preparation; unstained.)

C, fragments of ovarioles of an imago of the same age as fig. 5, E, showing formation of the pedicel. (Whole, fresh preparation; unstained.)

D, the terminal expanded part of the pedicel in longitudinal section, to show mucopolysaccharides in the epithelial cells. (Same treatment as fig. 5, E.)

E, the terminal expanded part of the pedicel in longitudinal section, to show glycogen. (Zenker; Bauer.)

are displaced to the periphery by the increasing mass of the ooplasm. Synthesis of lipid occurs subsequently in the superficial zone, near the acidophil layer; the lipid material extends from there towards the axial region.

The process of lipid yolk formation affirms the physiological unity of the primitive 13-cell group which in its posterior part (oocyte) differentiates as a yolk-elaborating centre, yet the primary lipid yolk synthesis begins in the common cytoplasm before its differentiation into nurse cells and oocyte.

With the growth of the oocyte a gradual apparent decrease in its RNA content is observed. This is the result of the RNA spreading over an increasingly larger volume of ooplasm. A basiphil zone persists for some time in the peri-nuclear space of the oocyte (fig. 3, D), but it soon disappears (fig. 5, B). The above fact proves that the oocyte itself produces no demonstrable RNA after separating from the group. All its RNA either derives from the common syncytial group or is conducted to it from the differentiated nutritive chamber.

The onset of the *flow of basiphil material* from the nutritive chamber initiates the second period of yolk formation. The basiphil stream penetrates through the narrow strand of cytoplasm connecting the oocyte with the nutritive chamber (fig. 5, B), and occupies the axial space of the oocyte.

Histochemical tests (methyl green / pyronin and Pappenheim's panoptic stain, both controlled by ribonuclease or perchloric acid digestion) reveal a high content of RNA in the stream. Basiphilia appears also after any basic dye. No DNA was detected after the Feulgen reaction (fig. 5, A), and no traces of polysaccharides after PAS or Bauer (fig. 5, E). Protein tests are negative (fig. 5, C), the protein component being probably masked by RNA. Histochemical tests show no difference between the composition of the basiphil strand and the cytoplasm of the nurse cells. The basiphil inflow persists till the conclusion of yolk formation, when the nurse cells have become inactive. In the final period of oogenesis the mass of RNA is displaced from its position by the accumulating yolk-mass and pushed forwards inside the pedicel. It disappears when the terminal extension of the pedicel is densely filled with other components.

Protein yolk synthesis starts soon after the basiphil material inflow appeared in the oocyte. The first protein spheres arise on the periphery of the inflow and fill the funnel-shaped space around it. Thence, as their mass increases,

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FIG. 5 (plate). A, part of ovariole of an immature imago in longitudinal section, showing the flow of RNA and the cytoplasm of the nurse cells (*pale*), and protein yolk (*dark*). (Zenker; Feulgen, light green.)

B, part of an ovariole of an imago slightly younger than A in longitudinal section, showing flow of RNA into the oocyte and absence of RNA in the perinuclear zone. (Zenker; Pappenheim.)

C, an oocyte in longitudinal section, showing protein yolk (dark). (Zenker; bromophenol blue).

D, ovariole in longitudinal section, showing lipids in the posterior nurse cells and in the lipocytes; intense synthesis in the oocyte. (Champy; unstained.)

E, a twisted ovariole in longitudinal section, showing mucopolysaccharides in the protein yolk region and in the pedicel epithelium. The oocyte on the left is the most regular. (Carnoy; P.A.S.)

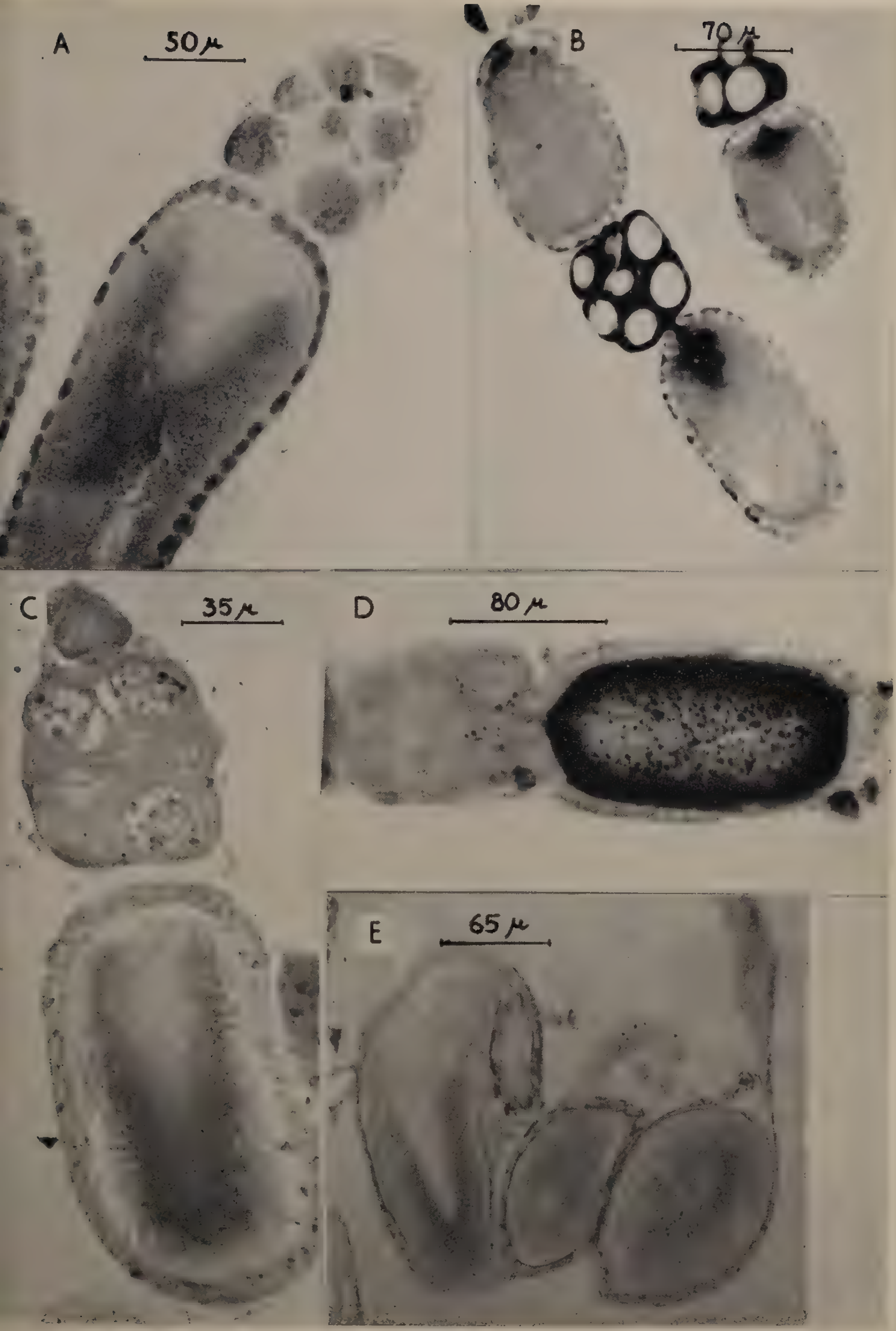

Fig. 5

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they spread towards the surface of the oocyte (fig. 5, A, C). Protein yolk components are acidophil and PAS-positive in all stages of yolk formation as well as in the mature egg. They fail to stain by the alcian blue technique. These results indicate that they are carbohydrate-protein complexes. Their carbohydrate compound is probably a neutral mucopolysaccharide, as usually found in insect tissues (fig. 5, E).

Glycogen cannot be detected until the final stage of yolk formation, when it appears as a delicate powder regularly dispersed all over the yolk-mass. A more strongly marked accumulation of glycogen is found in the anterior pole of the oocyte and in the pedicel, especially in its terminal extension (fig. 4, E). The glycogen particles tend here to fuse and produce big lumps. The glycogen picture varies in different preparations and appears to depend on fixation.

Protein bodies. In the initial period of differentiation a small, spherical, acidophil body appears in the ooplasm. Its formation may easily be followed after any RNA test, as it gives a bright contrast with the basiphil cytoplasm. Sometimes several small bodies are present. As the oocyte shifts backwards in the group, the acidophil body follows the germinal vesicle to the posterior part of the group. Another similar body appears somewhat later, when further differentiation of the oocyte is occurring (fig. 3, D). No similar structures remain in the cytoplasm of the nurse cells. The diameter of the body varies from 2 to 6 μ and is not correlated with the age of the oocyte. The protein bodies are the most acidophil component of the oocyte and give the most conspicuous result after all protein tests. Nucleic acids, glycogen, lipids (fig. 3, B), and mucopolysaccharides have not been detected in them. They differ from the protein yolk elements by being highly refractive in the living as well as in the fixed cell, and by the absence of polysaccharide. They are surrounded by a thin rim of RNA and by an unstainable zone outside this. They are soluble in acetic acid and consequently are absent in material fixed in Carnoy. In the growing oocyte, before yolk formation sets in, the two protein bodies take up a polar position like the centrosomes in the mitotic figure (figs. 2, D; 3, A, B). In the course of yolk formation they are pushed forwards by the growing yolk-mass to the anterior pole of the oocyte, near the connective strand of cytoplasm. Subsequently the protein bodies together with the anterior ooplasm are displaced into the terminal extension of the pedicel. There they give rise to the big protein spheres which fill the axial space of this structure (fig. 4, D). It follows from the above observations that the protein bodies arise in the cytoplasm of the cell group and their origin is not directly connected with any detectable activity of the nucleus or nucleolus of the oocyte or of the nurse cells. They show a striking resemblance to the nucleolus in their size and staining reactions but differ in the absence of RNA.

The egg follicle. The cells of the proliferating and growing follicular epithelium contain RNA (figs. 3, D; 5, B), but this disappears when growth ceases and the oocyte begins the second stage of vitellogenesis. In the epithelial cells of the maturing oocyte, when production of the egg envelope begins, accumulations of PAS-positive granules appear (fig. 5, E). They are most abundant in the epithelial cells coating the terminal extension of the pedicel (fig. 4, D). This part of the epithelial layer produces a thick envelope (fig. 4, C, E); it persists longer as an active tissue than the epithelium over the oocyte itself. No such envelope is formed on the surface of the nutritive chamber, which is devoid of a regular epithelial coating. The few cells, which are a remnant of the epithelium of the primary nutritive chamber, reveal neither RNA nor mucopolysaccharide and produce no envelope layer. A small amount of lipid (fig. 3, B) and of glycogen is always found in the follicle cells. The envelope of the *Cynips* egg is acidophil and PAS-negative, and gives a positive iodine test. These results show that it is epicuticular. No evidence was found of any secretory activity in the follicular epithelium which might indicate its contribution to yolk production. Secretion of the envelope layer seems to be the only demonstrable function of the follicular tissue.

Haemocytes. Two types of cells originating from the cellular elements of the haemolymph were found associated with the *Cynips* ovariole: fusiform cells of the external membrane of the ovariole (amoebocytes according to Wigglesworth's terminology), and lipocytes. Fusiform amoebocytes are haemolymph cells which primarily applied themselves to the gonad rudiment as described above. Their thin, flattened body contains PAS-positive granules, not revealed by the alcian-blue technique and presumably consisting of neutral mucopolysaccharide. RNA and lipids were not detected in them. Lipocytes penetrate inside the gonad in the early period of ovariolar differentiation, mainly in the oviduct region. They migrate forwards between the outer layer of amoebocytes and the epithelium, producing temporarily an epithelium-like layer (fig. 3, A). Finally, lipocytes accumulate round the interfollicular constriction separating the oocyte from the nutritive chamber (figs. 3, B; 4, A), producing a ring of about 10 cells between the epithelium and the external envelope (fig. 3, C). Lipocytes are small, spherical, or amoeboid cells with a slightly basiphil cytoplasm containing mucopolysaccharide granules, rich in lipids (fig. 5, D). Their constant localization on every ovarile indicates that their presence is not fortuitous but associated with function. Lipocytes are not in contact with the oocyte and their contribution to yolk formation can be excluded. No evidence was found for their phagocytic activity. Their role in elaboration of mucus is suggested by the presence of mucopolysaccharides in their cytoplasm.

DISCUSSION

The oogenesis of gall-flies was studied by Doncaster (1910, 1911, 1916) in *Neuroterus*, by Hogben (1920) in *Cynips kollari* and *Rhodites rosae*, and by Dodds (1939) in *Neuroterus buccarum*. These investigations were mostly concerned with meiosis and sex-determination, and contained only fragmentary data about the development of the ovariole, but the formation of cell groups was traced. According to Paulcke's conception, cells of the group were supposed to originate from one primitive germ cell. No histochemical study has previously been made of oogenesis in *Cynips*.

The activity of nurse cells and protein synthesis. The streaming of a basiphil substance from the nutritive chamber to the oocyte was reported in the early researches on insect oogenesis, and a role in the formation of yolk was suggested. The basiphilia of the streaming material suggested its origin from nuclear or nucleolar extrusions. The nature of the basiphil substance in growing cells and in those that elaborate a protein secretion was not satisfactorily elucidated until its relation to RNA was known. The idea that the basiphil material of cytoplasm contained RNA and that this was involved in the mechanism of protein synthesis, changed the outlook on the origin of yolk. Protein yolk was recognized as a product of the activity of RNA. In the *Cynips* ovariole the two processes—synthesis of RNA and synthesis of yolk—are dissociated in two different kinds of cells, and this makes the material favourable for the study of protein yolk synthesis.

The site of synthesis of cytoplasmic RNA is not known. Results of recent research suggest that RNA or its precursors are either synthesized in the nucleus or their synthesis is closely linked with it. This conception is based on results of research with the aid of autoradiographic and biochemical techniques. The autoradiographic study of Zalokar (1960) on the polytrophic ovary of *Drosophila* proved that the labelled RNA precursor ^{3}H uridine was first incorporated into the nurse-cell nuclei, which remained the most radioactive component of the cell. ^{3}H amino-acid was found especially in the cytoplasm. The author concludes that in the nurse cell the nucleus is the main site of RNA synthesis and the cytoplasm is the main site of protein synthesis. In the ovariole of *C. folii* the intense production of RNA during the life-cycle of the nurse cells and the lack of such production of RNA in the oocyte indicate that the nuclei of the nurse cells and not the germinal vesicle are responsible for RNA synthesis.

The methods applied in this study failed to reveal evidence of any passage of materials from nucleus to cytoplasm in the nurse cells or in the oocyte which might be interpreted as the transfer of RNA or its precursors from nucleus to cytoplasm. Electron-microscope studies provide some evidence that such interchange actually takes place. Anderson and Beams (1956) produced such evidence in their study of the nurse cells of *Rhodnius*. A Feulgen-negative substance, presumably RNA, was found penetrating the nuclear membrane. Using histological methods, Hsu (1953) found a material emitted by the nurse-cell nucleoli—but not by the germinal vesicle—in *Drosophila*. RNA was not shown to exist in this material, which proved to be a precursor of yolk granules. In the polytrophic ovariole of *Bombus* (Palm, 1948) and of *Apis* (Morgenthaler, 1952), the flow of RNA from the nurse cells was found responsible for the formation of protein yolk. Bier (1955) states, however, that in *Apis* the synthesis of protein yolk is independent of nurse-cell RNA, which contributes only to the synthesis of enzymes and structural protein.

Most authors (Peacock & Gresson, 1928; Dodds, 1939; Brunold, 1957) report the penetration of nurse-cell nuclei into the oocyte in the polytrophic ovariole of insects. The nuclei are supposed to be resorbed and utilized by the growing oocyte. The results of studies of the oogenesis of different insects are conflicting. In *Miastor* (Kahle, after Depdolla, 1926) the nutritive chamber persists outside the egg even during cleavage. In *Habrobracon* (Henschen, 1929), in *Bombus* (Palm), and in *Drosophila* (King & others, 1956), the life-cycle of the nurse cells is the same as in *C. folii*. The few cases of the penetration of nuclei in *Cynips* observed in the present study should be looked upon as an abnormality or artefact. Nevertheless, it shows the remarkable elasticity of the passage between the two parts of the former syncytial group, permitting 12 big nuclei to penetrate into the oocyte without damaging either the egg cell or the follicular epithelium. Resorption of such a considerable mass—if it were a normal process—would require the growing oocyte to be powerfully phagocytic, and this has not been proved. It must be concluded that in the ovariole of *C. folii* the nurse cells act as a mechanism elaborating RNA and not as a mass directly utilized for the synthesis of the oocyte components.

No substances other than RNA have been found streaming from the nurse cells to the cytoplasm of the oocyte. Certain facts seem to indicate that all the soluble material from the haemolymph (amino-acids, salts, sugars, &c.) which could not be detected by histochemical methods in this study, follow the same path through the nutritive chamber to the oocyte. The nurse cells are in a close contact with the haemolymph, whereas the oocyte is separated from it by the dense superficial layer of the ooplasm and the follicular epithelium. The experiments of Mieschtscherskaja (1931) on the permeability of the insect ovariole proved that the superficial layer of the young oocyte acts as a barrier to the penetration of soluble material. The experiments with vital staining in the present study support Mieschtscherskaja's results. Stains penetrate into the living oocyte only through the nutritive chamber, at least in the early stages.

According to the histochemical study of Bonhag on the ovary of *Oncopeltus*, lipids are supplied to the oocyte by the nutritive chamber, and in *Anisolabis* also glycogen and carbohydrate-protein complexes are supplied in the same way.

The follicular epithelium. Several functions of the follicular cells have been stated: yolk elaboration was observed by Gresson (1930) in Tenthredinidae, by Palm in *Bombus*, by Bier in *Apis*. Transmission of soluble material has been discussed above. In *Cynips* the secretion of epicuticular matter seems to be the most essential if not the only activity of this epithelium.

Protein bodies. Similar structures were observed by many authors in the insect oocyte but no histochemical tests have previously been tried to determine their nature. Basiphil bodies located in the same positions as in *C. folii* were found by Hogben (1920, fig. 96) in the oocytes of *C. kollari*. Structures of similar nature are known to occur in the oocyte of *Parascaris equorum* as the 'hyaline spheres' of van Beneden. They have recently been examined in detail both by cytochemical and biochemical methods by Fauré-Fremiet and his co-workers (1954). The spheres differ by their basiphilia but give results of histochemical tests consistent with those found in *C. folii*. They proved to be protein bodies containing no other components except some unidentified mineral material. Their role is unknown. The function of protein bodies in *Cynips* cannot be understood until the fate of the terminal pedicel extension in which they are localized is known. As yet little work has been done on early development of the gall-fly egg, because difficulties are encountered in finding the egg before the gall has appeared, and the development is then already far advanced.

The external sheath of the ovariole represents the connective tissue originating from cellular elements of the haemolymph. According to Wigglesworth (1956), these tissues are little differentiated in insects: the same kind of cells may function as free blood-cells and as fibroblasts. A *ring of lipocytes* in the constriction between the oocyte and the nutritive chamber has not previously been described. The presence of blood-cells associated with the ovariole is mentioned by Brunold (1957) in her study of the ovary of *Solenobia*.

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Histochemical Observations on the Succinic Dehydrogenase and Cytochrome Oxidase Activity in Pigeon Breast-muscle

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With two plates (figs. 1 and 2)

SUMMARY

Histochemical and cytochemical observations were made on the exact localization and distribution pattern of succinic dehydrogenase system and cytochrome oxidase in the pigeon breast-muscle by employing slightly modified methods. Succinic dehydrogenase activity, which was not detected earlier either histochemically or biochemically in the broad white fibres, was demonstrated by using a modified incubation medium under strictly anaerobic conditions, with neotetrazolium as the hydrogen acceptor. The size, localization, and distribution pattern of the histochemically demonstrable diformazan and indophenol blue granules showed a more or less close resemblance to the mitochondrial staining in the individual red as well as white fibres. The occurrence of high oxidative metabolism in the narrow red fibres was revealed by the presence of a large number of succinoxidase-positive granules in these fibres. On the other hand, the presence of fewer, smaller granules indicated very low oxidative metabolism in the broad white fibres.

The presence of the fewer, smaller succinoxidase-positive granules in the broad white fibres nevertheless shows that these fibres too possess mitochondria where at least a certain amount of oxidative activity does take place, and that they are to be considered as analogous to the white fibres of the other vertebrate skeletal muscles. It is also suggested that these granules are to be considered as mitochondria in the general sense and that the distinction between sarcosomes and mitochondria as proposed by previous authors needs reconsideration.

INTRODUCTION

IT has been shown (George & Jyoti, 1955; George & Naik, 1958*a*, 1958*b*, 1959) that the pectoralis major muscle of the pigeon consists of two distinct types of fibres, a broad, glycogen-loaded white variety with few mitochondria, and a narrow, fat-loaded, red variety having a large number of mitochondria in them. These two types of fibres existing side by side in one and the same system attracted our special attention. They have become the subject of more extensive studies with a view to understand their mutual relationship and mode of action.

In recent years the pigeon breast-muscle has been extensively used by biochemists and physiologists for studies in cell metabolism. What is known from these studies applies to the muscle as a whole and not to its separate components. Quantitative biochemical investigations on the two types of

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fibres is by no means easy, since the complete isolation of these fibres without damage appears extremely difficult if not impossible. Studies on the relative distribution and localization of metabolites and enzymes in these fibres by histochemical and cytochemical methods were therefore undertaken in our laboratories. George and Scaria (1956) demonstrated the presence of a lipase in this muscle. George and Iype (1960) using improved histochemical method showed that lipase is localized in the red, fat-loaded fibres. George, Nair, and Scaria (1958) showed that alkaline phosphatase is mainly confined to the same fibres, while George and Pishawikar (1959) have shown a higher concentration of ATPase in the white, glycogen-loaded fibres.

George and Scaria (1958) in a histochemical study of dehydrogenases (succinic, malic, lactic, and glycerophosphate dehydrogenases) could not detect any of these in the broad white fibres. They suggested that these fibres in the pigeon breast-muscle are a unique system in which none of the oxidative processes concerned with the above enzymes takes place, and that these fibres therefore cannot be considered as analogous to the white fibres of the other vertebrate skeletal muscles studied. The method employed by these authors was that of Straus and others (1948), who used the colourless salt, triphenyl tetrazolium chloride, as the hydrogen acceptor. George and Talesara (1960), employing the method of Kun and Abood (1949), who used TTC under aerobic conditions, studied quantitatively the distribution pattern of succinic dehydrogenase in the different layers of the pigeon breast-muscle and concluded that the main bulk of the enzyme is restricted to the narrow red fibres. These observations on the succinic dehydrogenase system in the pigeon breast-muscle, made by histochemical and biochemical methods, were recorded under aerobic conditions. Within the limitations of the methods employed no significant enzyme activity was detected in the broad white fibres.

It is well known that in a living system the transport of hydrogen from succinate in the presence of the succinic dehydrogenase system brings about the reduction of cytochrome C. This reduced cytochrome C is in turn oxidized by the molecular oxygen in the presence of cytochrome oxidase, which is the terminal enzyme in the entire respiratory chain. It was therefore thought necessary to examine the distribution of cytochrome oxidase in the two types of fibres. The classical 'Nadi' reaction was employed for the histochemical demonstration of the localization of the enzyme. The muscle sections thus treated revealed the presence of a detectable concentration of cytochrome oxidase in the broad white fibres. The results thus obtained naturally questioned the validity of the earlier observations made regarding the absence of the succinic dehydrogenase activity in the broad white fibres, and also regarding the specificity of the methods employed, both histochemical and biochemical.

This stimulated us to seek a more specific and sensitive method for the detection of the succinoxidase system, so that it would be possible to detect histochemically even small concentrations of the enzymes. We were able to improve the usual methods. Under strictly anaerobic conditions it was possible to demonstrate succinic dehydrogenase activity in the broad white fibres, which could not be done with the ordinary methods. In this paper we report the distribution and the localization of succinic dehydrogenase and of cytochrome oxidase by improved and modified histo-chemical methods.

Material and Methods

In order to ensure that only uniformly well-developed pectoralis major muscle was used, well-fed and fully grown, normal laboratory pigeons (*Columba livia*) of both sexes weighing between 280 to 340 g were used throughout.

Histochemical localization of the succinic dehydrogenase system

The activity of the succinic dehydrogenase system in the narrow red fibres could be demonstrated easily by the ordinary histochemical methods. But the broad white fibres in which the concentration of this enzyme is extremely low requires a more sensitive and improved method of treatment. The specificity and the sensitiveness of the method in turn would depend upon a number of factors. Several authors (Straus & others, 1948; Black & Kleiner, 1949; and Black & others, 1950) used triphenyl tetrazolium chloride as the hydrogen acceptor for the demonstration of succinic dehydrogenase in animal tissues. Later, Seligman and Rutenburg (1951), Padykula (1952), Rutenburg, Wolman, and Seligman (1953), and Whitehead and Weidman (1959), in their modified methods for the histochemical localization of succinic dehydrogenase in various tissue sections, reported that under anaerobic conditions the tetrazolium reduction was more rapid and intense, and some tissue sections which were not stained under aerobic conditions were stained under anaerobic conditions. The same observation was made by Kun and Abood (1949), Brodie and Gots (1951), Cooper (1955), and Padykula (1958) in the biochemical determination of succinic dehydrogenase. Of the various tetrazolium salts used, TTC, blue tetrazolium (BT), and neotetrazolium (NT), the latter was found to be most suitable for demonstrating succinic dehydrogenase activity histochemically. It has also been preferred by Shelton and Schneider (1952), Padykula (1952, 1958), Pearse (1954), Farber, Sternberg, and Dunlap (1956*b*), and Cascarano and Zweifach (1955). Rosa and Velardo (1954), in a modified technique, suggested the incorporation of sodium cyanide into the incubation medium as an effective blocking agent for cytochrome oxidase and also as a trapping agent for any oxaloacetic acid that may be produced during the processing of the sections.

The whole literature on the histochemical methods employed for the demonstration of DPN diaphorase, TPN diophorase, and the succinic dehydrogense system has been reviewed critically in a series of papers (Sternberg, Farber, & Dunlap, 1956; Farber, Sternberg, & Dunlap, 1956*a*; Farber, Sternberg, & Dunlap, 1956*b*; Farber & Louviere, 1956; Farber &

Bueding, 1956). In a study of the histochemical localization of specific oxidative enzymes, Farber and Louviere (1956) showed that the addition of any one of a number of soluble redox dyes to the incubation medium serves as a carrier between the enzyme and the tetrazolium salt, thereby increasing the rate of staining and making it possible to demonstrate the presence of enzymes which do not react directly with tetrazolium. Nachlas and others (1957) devised a simple method by the use of new tetrazole (nitro-blue tetrazolium) for the cytochemical demonstration of succinic dehydrogenase system. Pearson (1958) used nitro-neotetrazolium chloride. We have, however, restricted ourselves to the use of neotetrazolium because of the very satisfactory results obtained with it. The TTC method of Straus and others (1948) was also tried for comparing the results obtained by our modified method which successfully demonstrated the presence of the succinic dehydrogenase system in the broad white fibres of the pigeon breast-muscle. Our modifications were mainly in the composition and use of the incubation medium. We based our methods on those reported by Seligman and Rutenburg (1951), Padykula (1952), Rutenburg, Wolman, and Seligman (1953), Rosa and Velardo (1954), Farber and Louviere (1956), and Farber, Sternberg, and Dunlap (1956*b*). The composition of the incubation medium used (in a total volume of 6 ml) was as follows:

PO_4 buffer (0·1 M, pH 7·6)	2·5 ml
Na succinate (0·5 M neutralized to pH 7·6)	0·6 ml
$CaCl_2$ (0·004 M)	0·5 ml
$AlCl_3$ (0·004 M)	0·5 ml
$NaHCO_3$ (0·6 M, freshly prepared)	0·3 ml
NaCN (0·03 M, brought to pH 7·6)	0·5 ml
Neotetrazolium (3 mg/ml, freshly prepared)	1·0 ml
$MgSO_4$ (0·005 M)	0·05 ml
Methylene blue (2 mg/ml)	0·05 ml

The incubation mixture was boiled in a small vial to remove dissolved oxygen. The vial was stoppered immediately. After cooling, the mixture was stored in a bath maintained at 37° C till the sections were ready for incubation.

For each experiment a bird was decapitated and bled. A piece of muscle 1 cm in length, consisting of the complete depth of the muscle, was immediately cut out and mounted on the stage of a carbon dioxide freezing microtome. The muscle-block was frozen hard as quickly as possible so as to avoid the formation of ice crystals and distortion of the tissue. Sections between 20 to 40 μ thick were cut and floated in ice-cold phosphate buffer for nearly 10 min in order to remove the endogenous substrates from dehydrogenase activity. The sections were then transferred quickly to the vial containing the incubation mixture. The method followed for creating the anaerobic conditions required for the experiment was that of Padykula (1952). The vial with Padykula's arrangement was placed in a bath maintained at 37° C. A gentle bubbling of nitrogen was continued during incubation. The incubation was carried out for 15 to 60 min as desired. After incubation the sections were washed thoroughly in phosphate buffer, fixed in 10% buffered neutral formalin at 4° C for 4 to 6 h, and after washing mounted in glycerogel. The preparations were immediately examined under a microscope and photographed.

In order to verify the specificity of the succinic dehydrogenase activity, two sets of control experiments were conducted. In one the sections from cold phosphate buffer were incubated anaerobically in the absence of succinate. In the other the sections were treated with malonate. Supplementary experiments were performed on frozen sections with TTC or NT as the hydrogen acceptor, under aerobic or anaerobic conditions, without the intervention of activators or the soluble redox dye.

To rule out the possibility of any false localization of succinic dehydrogenase activity resulting from diffusion, which is liable to occur in a mixed muscle like the pigeon pectoralis where the two types of fibres exist side by side, a simple technique was followed. In each experiment a small strip of muscle from a freshly killed pigeon was cut and transferred to a 0·9% cold NaCl solution. Immediately after this the two types of fibres from the mixed muscle were isolated by teasing them out by a pair of fine watchmaker's forceps under a stereoscopic dissection microscope and treated separately for the demonstration of succinic dehydrogenase activity by the method mentioned above.

Histochemical localization of cytochrome oxidase

The classical Nadi reaction, employing a mixture of *a*-naphthol and dimethyl-*p*-phenylene diamine hydrochloride, demonstrates the presence of cytochrome oxidase in cells (Moog, 1943; Borei & Bjorklund, 1953). A critical review of the merits and demerits and the specificity of the Nadi reaction for the histochemical demonstration of cytochrome oxidase has been given by Gomori (1953), Pearse (1954), Crawford and Nachlas (1958), Nachlas, Crawford, Goldstein, and Seligman (1958), and Burstone (1959). We followed a method adopted from the reports of Moog (1943) and Nachlas, Crawford, Goldstein, and Seligman (1958) for demonstrating histochemically cytochrome oxidase in fresh frozen sections of the pigeon-breast muscle. The Nadi mixture used was composed of the following reagents:

PO_4 buffer (0·1 M, pH 7·4)	3 ml
a-naphthol (1 mg/ml in 1% NaCl)	5 ml
Dimethyl-*p*-phenylene diamine hydrochloride (1 mg/ml in 1% NaCl)	5 ml
Cytochrome C (3 mg/ml)	2 ml

All the reagents were freshly prepared just before use, mixed together, and filtered. Cytochrome C was omitted from the medium in the routine experiments unless required specifically. Addition to the incubation medium of a catalase and an inhibitor for dehydrogenase was not found necessary.

As mentioned in the case of the succinic dehydrogenase system, fresh frozen sections of the muscle were placed quickly in 0·9% cold NaCl solution and then incubated in the freshly prepared Nadi mixture at room temperature for 5 to 15 min as required. Control sections were treated with sodium azide and sodium cyanide as recommended by Moog (1943). After incubation, sections were washed thoroughly in 0·9% NaCl and mounted in saturated potassium acetate. Photomicrographs were taken immediately. It was observed, however, that the sections mounted in potassium acetate and maintained at 4° C, lasted for a number of days without any visible change in colour or pattern of granules.

As mentioned in the case of succinic dehydrogenase, the two types of fibres were isolated and treated separately for cytochrome oxidase activity. In order to compare the histochemically demonstrable granular pattern of the succinic dehydrogenase system and cytochrome oxidase with mitochondrial staining, sections of the pigeon breast-muscle were stained by Janus green B and also by Altmann's aniline fuchsin method as described by Gray (1954).

OBSERVATIONS

Distribution of succinic dehydrogenase system

When frozen sections were incubated aerobically without the addition of activators or soluble redox dye, with TTC as hydrogen acceptor, a regular distribution pattern of the succinic dehydrogenase system was observed in the narrow red fibres. These red fibres were stained within 10 min while the broad white fibres did not show any detectable activity of succinic dehydrogenase even after incubation for 1 h (fig. 1, D). But when the sections were treated with the modified incubation medium under strictly anaerobic conditions, with NT as indicator, the localization of the enzyme in both types of fibres was observed at the cytochemical level (fig. 1, A, B). Succinic dehydrogenase activity was revealed in the red narrow fibres within 5 to 10 min as fine bluish-violet diformazan granules distributed in a most regular pattern in both transverse and longitudinal sections. After an incubation period of 15 to 30 min the presence of succinic dehydrogenase was revealed in the white fibres by lighter bluish-violet diformazan granules, which were, of course, smaller and far fewer than those of the narrow red fibres (fig. 2, A, B). A clear picture of the exact distribution of the granules was obtained in longitudinal sections, where red and white fibres were side by

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FIG. 1 (plate). A, longitudinal section passing through the broad white fibre of the pigeon breast-muscle, showing the distribution and localization pattern of histochemically demonstrable succinic-dehydrogenase-positive granules arranged linearly along the myofibrils. The granules are far fewer and smaller than in the narrow red fibres. Incubation period 1 h.

B and C, longitudinal section through the narrow red fibres of the pigeon breast-muscle, showing the histochemically demonstrable succinic-dehydrogenase-positive granules arranged linearly along the myofibrils and representing more or less the distribution pattern of mitochondria. Incubation period 15 min.

D, transverse section of pigeon breast-muscle showing the distribution of succinic dehydrogenase in the two types of fibres, treated under aerobic conditions with triphenyl tetrazolium chloride. Note the unstained broad white fibre. Incubation period 1 h.

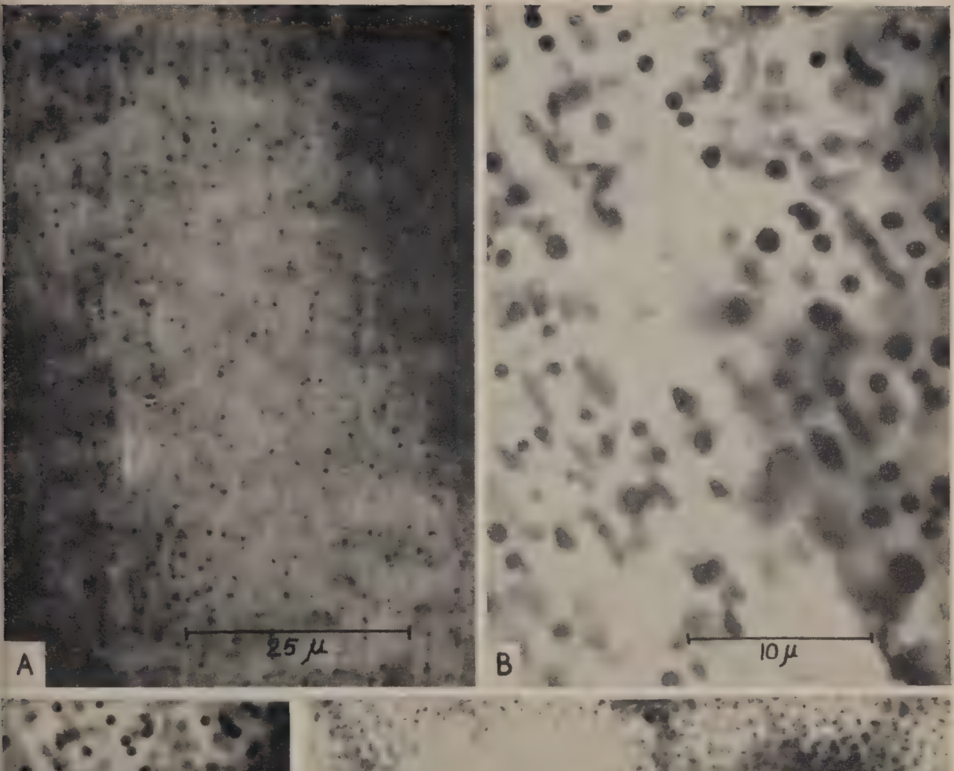

Fig. 1

J. C. GEORGE *and* C. L. TALESARA

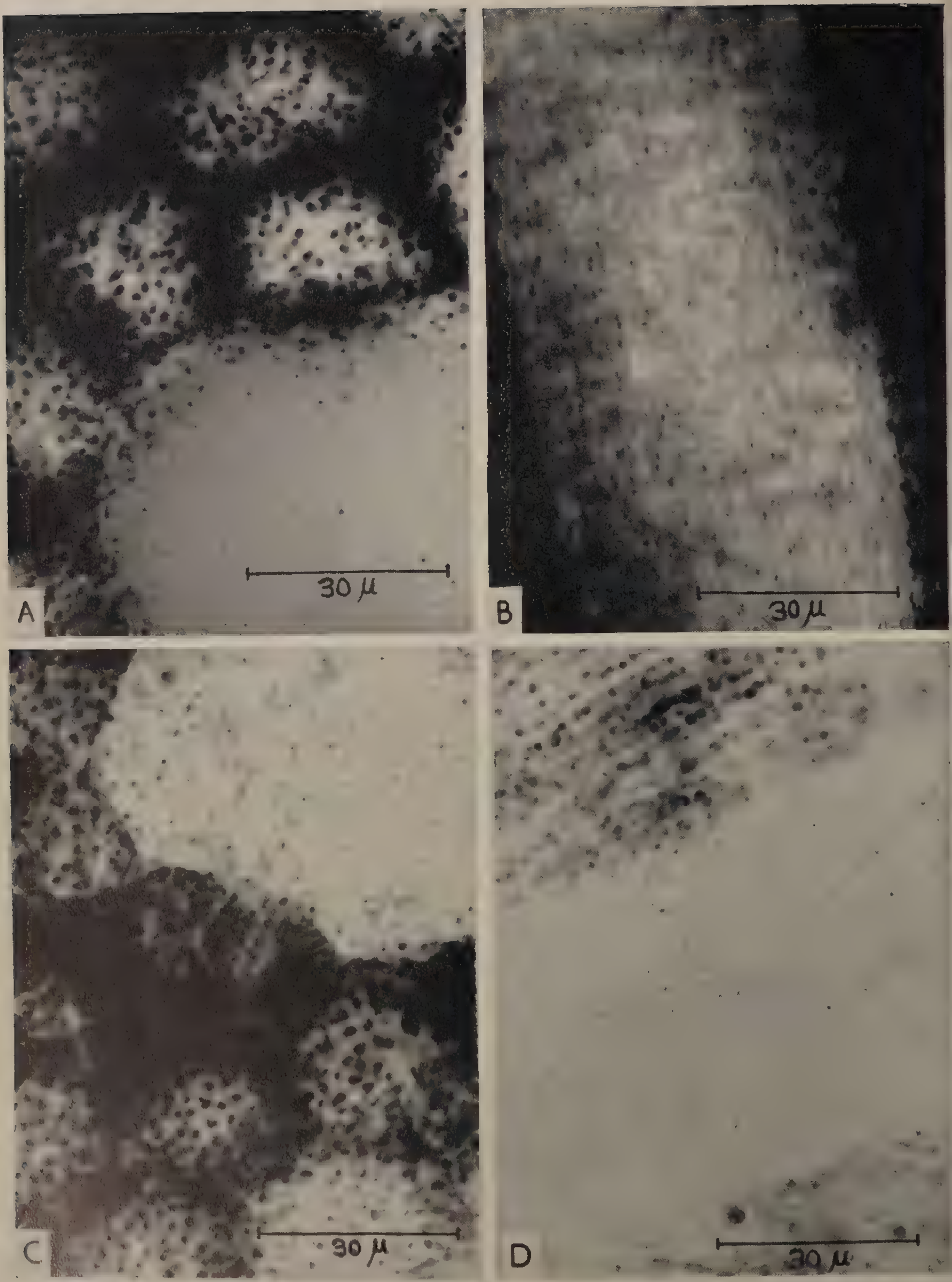

FIG. 2

J. C. GEORGE *and* C. L. TALESARA

side. The sarcoplasm of the muscle-fibres was stained slightly pink and also contained sharp bluish-violet diformazan granules, which were arranged in a definite linear fashion between the unstained myofibrils (fig. 1, A–C). The localization of the succinic dehydrogenase system, which could not be demonstrated in the broad white fibres by ordinary methods, thus became visible (table 1).

In addition to the diformazan granules in the sections, a diffused red colour was visible in most of the narrow red fibres. This probably represented the partially reduced form of the dye (monoformazan), or the dye dissolved in the fat which is quite abundant in the narrow red fibres. The inter-fascicular fat was stained uniformly deep red and no granules were visible. Thus the sharp diformazan granules with the distinct bluish-violet colour showed the exact sites of the localization of the succinic dehydrogenase system in the two types of fibres (fig. 2, A).

TABLE 1

Histochemically demonstrable succinic dehydrogenase in the broad white fibres of the pigeon breast-muscle

Expt.	*Incubation mixture in a final volume of 6 ml; pH 7·6*	*Degree of staining in 1 h*	
		aerobic	*anaerobic*
1	Medium A TTC+succinate+PO_4 buffer	O	±
2	Medium B NT+succinate+PO_4 buffer	+	++
3	Medium C NT+succinate+PO_4 buffer +activators (Ca^{++}, Al^{+++}, MG^{++}, and HCO_3^-)+NaCN	++	+++
4	Medium D Reagents of medium C+methylene blue	++	++++

O = no detectable staining;
± = trace of detectable staining;
+, ++, +++, ++++ = relative degrees of staining under different conditions.

Fresh frozen sections, at different levels in the whole depth (ventral face to dorsal face) of the pigeon breast-muscle when treated for the succinic

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FIG. 2 (plate). A and B, transverse and longitudinal sections respectively of pigeon breast-muscle, showing the distribution and localization pattern of succinic dehydrogenase in the two types of fibres. A modified incubation medium was used under anaerobic conditions. The narrow red fibres show a markedly greater deposition of diformazan granules than the broad white fibres. Note the smaller diformazan granules in the broad white fibre. Incubation period 30 min.

C and D, transverse and longitudinal sections respectively of pigeon breast-muscle, showing the localization and distribution pattern of cytochrome oxidase. Note the similarity in the size, distribution, and localization pattern of indophenol blue granules with that of the diformazan ones (A and B). Incubation period 10 min.

dehydrogenase system, showed that in each individual narrow and broad fibre, the degree of staining for the enzyme was more or less the same in the corresponding fibres throughout the depth of the muscle. In the narrow red fibres relatively more granules were observed towards the periphery. TTC was not found suitable for the low enzyme activity in the broad white fibres because of the diffused pale red colour it formed during enzymic reduction, which did not give a sharp contrast under the microscope. On the other hand, neotetrazolium, because of the sharp blue-violet granules it formed, was found more suitable for such studies.

Distribution of cytochrome oxidase

The distribution pattern of cytochrome oxidase by the Nadi reaction showed a close resemblance in all respects to that of succinic dehydrogenase. Cytochrome oxidase activity was indicated by the formation of indophenol blue granules resulting from the oxidative coupling of *a*-naphthol and dimethyl-*p*-phenylene diamine hydrochloride in the presence of cytochrome C, in both the narrow and the broad fibres of the pigeon breast-muscle (fig. 2, C, D). The diffusibility and the solubility in fat of the final dye, indophenol blue, was observed in the form of a uniform violet colour. This violet staining could easily be distinguished from the actual sites of the localization of cytochrome oxidase in the form of dark blue indophenol granules. The interfascicular fat was also stained deep violet. During a short incubation period without added cytochrome C no detectable colour was observed in the broad white fibres. However, blue granules were seen in these fibres when cytochrome C was introduced into the incubation medium.

The two types of fibres isolated under a dissecting microscope and separately treated histochemically for succinic dehydrogenase and cytochrome oxidase, showed the same distribution pattern of the granules as was observed in the sections.

The longitudinal and transverse sections of the pigeon breast-muscle stained for mitochondria, succinic dehydrogenase, and cytochrome oxidase all showed close similarity as regards the actual sites of staining. On careful observation it was found that the size and distribution pattern of the diformazan as well as indophenol blue granules more or less coincided with those of the mitochondria.

DISCUSSION

The above observations have shown that the two types of muscle-fibres in the pigeon pectoralis, when treated for succinic dehydrogenase and cytochrome oxidase, stain with different intensity. A distinct pattern of granules representing the succinoxidase system in the sarcoplasm between myofibrils more or less coincides with the pattern of the mitochondria. It can therefore be concluded that the actual sites of the localization of the succinoxidase system is in or near the mitochondria, and the histochemically demonstrable diformazan and indophenol blue granules do represent the pattern of mitochondrial distribution in pigeon breast-muscle. It is well known that succinic dehydrogenase may be demonstrated histochemically in or near the mitochondria (Goddard & Seligman, 1952, 1953; Nachlas & others, 1957; Scarpelli & Pearse, 1958). The same applies in the case of cytochrome oxidase, as the activity of the succinic dehydrogenase system is linked with the cytochrome system. The considerably less succinoxidase activity observed in the broad white fibres is obviously due to their low mitochondrial content. This contention is supported by both biochemical (Hogeboom, Schneider, & Palade, 1948; Harman, 1950; Paul & Sperling, 1952; Harman & Osborne, 1953; Chappell & Perry, 1953) and electron microscopic studies (Barnett & Palade, 1957; Sedar & Rosa, 1958), which have shown that succinic dehydrogenase is exclusively present in the mitochondria. The granules thus made visible in the broad white fibres of the pigeon breast-muscle are to be regarded as mitochondria. These have not been reported by previous workers.

The narrow red fibres possess a large volume of sarcoplasm and numerous granules of large size (fig. 1, B, C), while the broad white fibres have considerably less sarcoplasm and possess much fewer, smaller granules (fig. 1, A). Further details regarding the actual size and exact position of these smaller succinoxidase-positive granules in the broad white fibres could only be made available by using the electron microscope. The myofibrils of both the red narrow and the broad white fibres do not show any detectable positive reaction for the succinoxidase system. The absence of this system in myofibrils has been shown biochemically by Harman and Osborne (1953) in a pure suspension of myofibrils.

In fresh, unfixed, frozen sections all granules representing sites of succinoxidase activity are spherical and of about the same size in fibres of the same type (larger in the red fibres than in the white). Various authors (Harman & Osborne (1953); Kitiyakara & Harman (1953); Weinreb & Harman (1955); Harman (1955)) have distinguished two types of cytochondria, the spherical forms as sarcosomes and the large elongated ones as mitochondria. Both are said to be linearly arranged along the myofibrils in the red fibres of the pigeon breast-muscle. In our fresh frozen preparations, however, we were able to detect only spherical granules in both types of fibres: the elongated ones (Harman's mitochondria) were not found (fig. 1, A, B). A similar picture to ours (fig. 1, B) was obtained by Harman also (fig. 1, 1955). His claim of the existence of the rodlet mitochondria does not appear convincing. Evidence from electron microscopy in support of his opinion (his figs. 2 and 3) does not appear to be convincing. We are inclined to believe that the spherical granules in both the types of fibres are to be regarded as mitochondria in the general sense.

When frozen sections were incubated in Nadi mixture with added cytochrome C, an increase in cytochrome oxidase activity was observed. This suggests that the broad white fibres possess a very low concentration of cytochrome C, whereas the narrow red fibres seem to be very rich in it. In a similar study Katchman and Shooter (1955) and Maruyama and Moriwaki (1957) have shown, in pigeon breast-muscle and in the thoracic muscle of the honey bee respectively, the dependence of succinoxidase activity on cytochrome C concentration. It seems probable that the low succinoxidase activity in the broad white fibres of the pigeon breast-muscle is associated with the higher glycogen deposition. Thus the characteristic glycogen load may be a result of poor oxygen supply, leading to anaerobic metabolism.

These observations throw some more light on the earlier observations made regarding the occurrence of oxidative enzymes in the two types of fibres. The presence in the white fibres of a few smaller granules, responding positively to tests for succinic dehydrogenase and cytochrome oxidase, indicates the existence of some sort of cytochondria in them with a very low oxidative metabolism, even less than that of the white fibres of the other vertebrate skeletal muscles. It should, however, be emphasized that the white fibres of pigeon breast-muscle are not fundamentally different from the white fibres of the other vertebrate skeletal muscles. On the contrary, it can be concluded from these studies that in such a mixed type of muscle as the pigeon pectoralis, the broad white fibres, chiefly loaded with glycogen and with very low concentrations of oxidizing enzymes, function mostly anaerobically and in all probability are meant for quick and sudden contraction for a short duration, whereas the narrow red fibres loaded with fat and with very high concentrations of the Krebs cycle enzymes, are used for sustained contraction.

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